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# Novel Approach to High Throughput Screening for Activators of Transcription Factors

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## 1. Introduction

There is growing body of evidence that many diseases require a systemic treatment approach rather than targeting just a single enzyme or receptor. Neurodegenerative diseases affect a wide spectrum of the population and, in most cases, lead to physical and/or mental incapacity, involving memory, cognition, language and personality. Aging contributes to the development of neurodegeneration by shifting the equilibrium between oxygen-dependent and independent mechanisms of energy production towards mitochondria-generated ATP. Mitochondrial capacity degenerates with aging, making cells susceptible to both ischemic and oxidative insults. Although all mechanisms of hypoxic preconditioning are very far from complete understanding, it is clear that cell is equipped with the necessary molecular machinery to respond to the changes in intracellular oxygen content. However, aging significantly compromises this response. Turning on the existent molecular machinery to compensate for hypoxic and oxidative stress may lead to comprehensive and safe therapeutic strategy for age-related neurodegenerative disorders.

To restore homeostatic balance destroyed as a result of acute or chronic injury, one needs to activate intrinsic genetic programs silent or insufficiently active in the damaged cells. Activation of cellular defensive systems requires stabilization of corresponding transcription factors which govern expression of cassettes of genes turning on a particular program such as antioxidant, anti-inflammatory, or anti-hypoxic one. Hence, one of the emerging strategies in drug screening becomes a hunt for activators (or inhibitors) of transcription factors. In some cases there is an enzyme responsible for a rate-limiting conversion of a transcription factor, in some not. The well-known and commonly used approach to screening of activators of transcription factors is the use of luciferase gene cloned under the promoter activated by a specified transcription factor. For example, cell-based screening for activators of hypoxia induced factor 1 (HIF1) is performed using HRE (hypoxia response element)-luciferase (Semenza et al 1996), and for Nrf2 – using ARE (antioxidant response element)-luciferase reporter assay (Moehlenkamp & Johnson 1999). Promega has just begun to offer commercially such type of constructs with any desired promoter for research and drug screening purposes.

Although these reporters are obviously of great help in research, they provide an integrated response and are affected by many steps such as transcription factor stabilization, phosphorylation, translocation to nucleus, interaction with transcription cofactors, etc. As a result, the response of these reporters is not immediate: it is delayed by many hours, the magnitude of activation effect is very modest, and there is always uncertainty in the actual site of their effect along the path leading to activation of a particular promoter.

The important general pathway effectively regulating the cellular response to hypoxia, oxidation and inflammation is specific regulation of protein stability of the corresponding transcription factors or their modulators by ubiquitination and proteasomal degradation. Protein degradation by the proteasome is one of the major regulatory mechanisms in the cell. The proteasome mediates the degradation of most short-lived proteins that control cell cycle, transcription, DNA repair, apoptosis and other cellular processes. Under normal conditions, the stress response transcription factors are expressed constitutively, but on the protein level, these transcriptional factors are unstable: they undergo ubiquitination either directly, or upon specific covalent modification(s) of the targeted amino acids residues (like hypoxia inducible factor, HIF). Considering the protein stability of transcription factors as the most important and limiting step in the stress response we have recently developed a different approach to high throughput screening (HTS) of stabilizers of transcription factors. The approach is based on stable expression of a fusion between luciferase and a transcription factor minimum domain recognized by ubiquitination machinery (Fig.1).

Minimum domain is the portion of a transcription factor that is necessary and sufficient for recognition and ubiquitination steps to occur. The overexpressed luciferase-labeled surrogate of a transcription factor undergoes the same recognition and transformation steps as an endogenous one. The time-course of reporter signal changes can be easily followed: the luminescent readings are extremely sensitive and provide monitoring just minutes after drug administration.

This new approach to HTS was developed in this laboratory and successfully used to discover novel activators of HIF1 and Nrf2 as exemplified below.

## **2. Development, validation and application of novel reporters for the purposes of HTS**

### **2.1 Neh2-luciferase reporter construction and performance (Smirnova et al 2011)**

The key transcription factor that orchestrates antioxidant response is Nrf2 (Moi et al 1994, Motoyashi & Yamamoto 2004, Kaspar et al 2009). Compounds that activate Nrf2 make the cell more resistant to subsequent xenobiotic attack or oxidative stress. This has major implications for human health: (1) Nrf2 activators can be considered as medications for cancer prevention; (2) while an increased level of Nrf2 makes cancerous cells more resistant to chemotherapy; and (3) Nrf2 activators can both prevent and treat neurodegenerative diseases. There is a consensus that oxidative stress either derived from gene mutations or environmental toxins is a mediator of neurodegenerative diseases and thus, Nrf2 has been justified as a pharmacological target for neuroprotective therapies in Parkinson's, Huntington's and other neurodegenerative diseases.

#### **2.1.1 Nrf2 - Keap1 interaction mode**

Nrf2 is composed of Neh1–Neh6 domains, among which Neh2 (1-98 aa) is the putative negative regulatory domain that interacts with Keap1, while Neh4 and Neh5 are

transactivation domains, and Neh1 is the binding domain for ARE. The functional domains of Keap1 are the Broad complex, Tramtrack and Bric-a-Brac, the intervening region, the double glycine repeats domain, and the C-terminal region (Zhang et al 2006). Two motifs in the Neh2 domain, i.e. ETGE and DLG (Tong et al 2006 a, 2006b), are recognized by the Keap1 homodimer in a hinge-latch mode (see Fig.2 and 3). Keap1 mediates polyubiquitination of the lysines positioned within the central  $\alpha$ -helix of the Neh2 domain under homeostatic conditions. Under oxidative/electrophilic stress, most reactive cysteines within Keap1 (Cys 151, Cys273, Cys288) are modified and Keap1 undergoes conformational changes which lead to Nrf2 stabilization (see Fig.3) (Cullinan et al 2004).

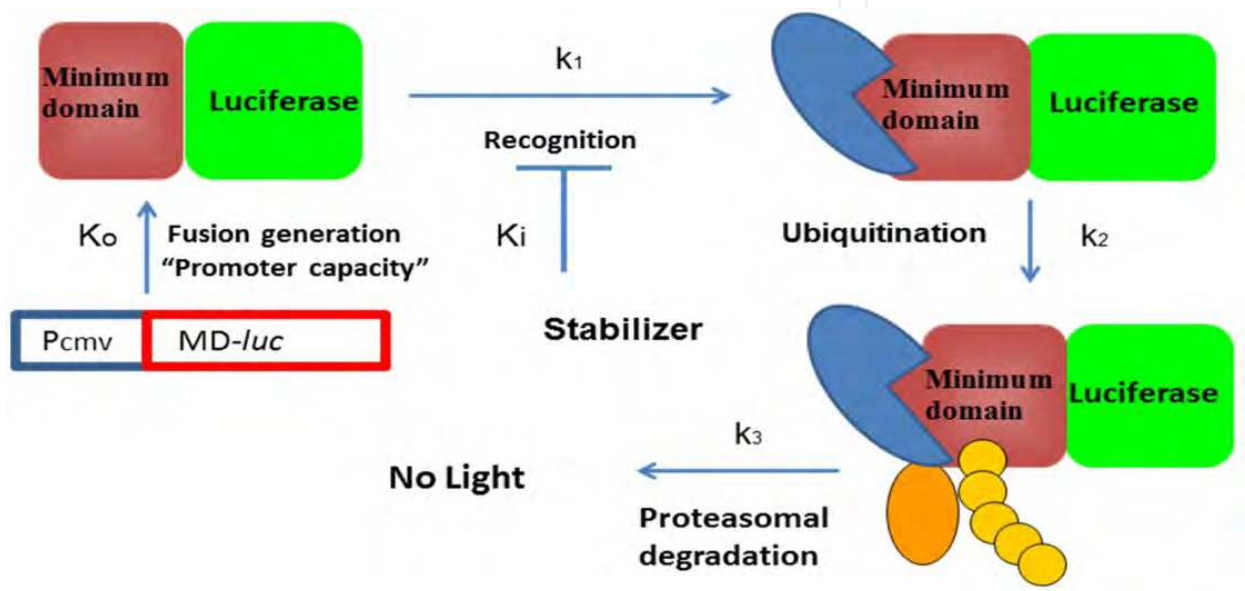


Fig. 1. Schematic presentation of reporter performance based on constitutive expression of luciferase-labeled minimum domain of a transcription factor. The background luminescent signal corresponds to the sum of all forms of labeled surrogate except for its proteolytic fragments:  $[MD-luc] = K_o \sum (1/k_i)$ . Specific stabilizers of a transcription factor must work at the recognition step: each reporter requires validation to demonstrate that this particular step is rate-limiting, meaning that  $[MD-luc] = K_o \sum (1/k_1)$ . If this is the case, HTS will select for specific stabilizers of a transcription factor working at the recognition step.

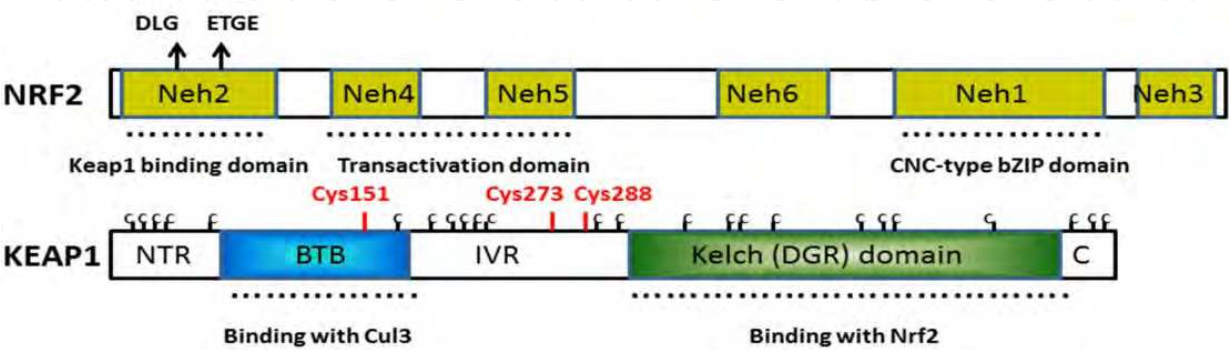


Fig. 2. Schematic presentation of domain structures of Keap 1 and Nrf2.



Nrf2 translocates to the nucleus, interact with Maf protein, and induces expression of the cytoprotective enzymes such as glutathione reductase, thioredoxin reductase, glutathione S-transferase (GST), hemeoxygenase-1 (HO1), catalase, etc. Alkylating agents are supposed to target Cys151 to detach Cul3 ubiquitin-ligase and inhibit ubiquitination (Zhang et al 2004). Keap1 thiols Cys273 and Cys 288 are supposedly responsible for dimer conformation capable of binding Nrf2: changes in Keap1 conformation disrupt dimeric structure and also result in inhibition of ubiquitination. The yet unknown specific stabilizers of Nrf2 should disrupt interaction between DLG domain of Nrf2 and Keap1 and thus prevent ubiquitination of Neh2 lysines and result in Nrf2 stabilization.

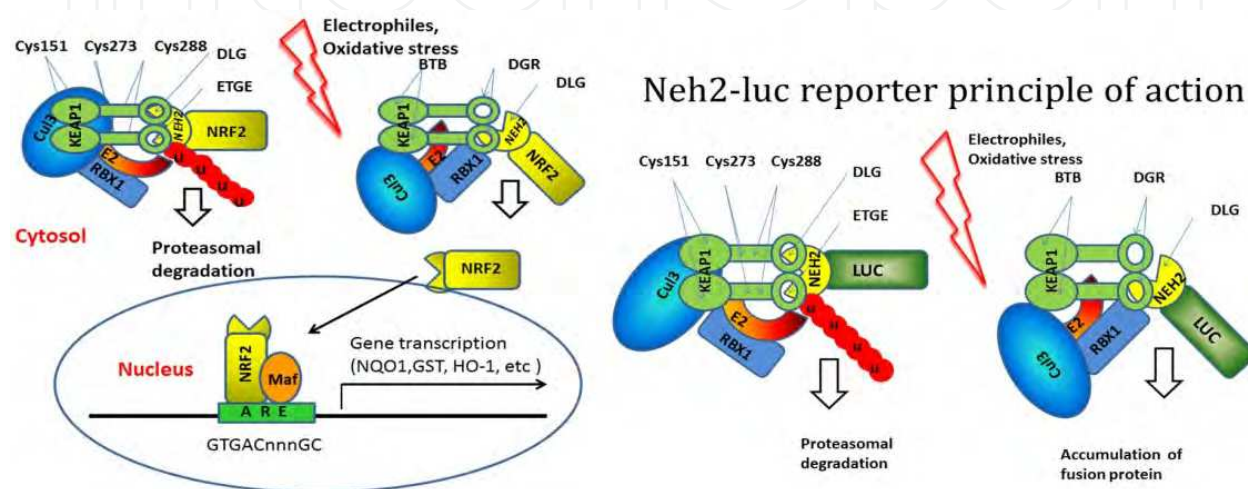


Fig. 3. Schematic presentation of Nrf2 stabilization and transcription activation (left) versus the performance of Neh2-luc reporter (right). Keap1-bound Nrf2 is subject to ubiquitination, and then undergoes proteasomal degradation. Upon oxidative stress, Keap1 thiols are either alkylated (Cys 151) resulting in detachment of Cul3 ubiquitin ligase or modified (Cys273 and Cys288) leading to a conformation change in Keap1 dimer and release of Nrf2 from the complex. In both cases Nrf2 protein is stabilized, and the same mechanism of stabilization applies to the Neh2-luc reporter performance. Luciferase labeling of Neh2 permits easy monitoring of fusion accumulation upon oxidative stress induced by small molecule activators.

### 2.1.2 Neh2-luciferase reporter validation

The  $P_{cmv}$ -driven Neh2-luc reporter supports the constitutive, intracellular synthesis of a novel fusion protein composed of the Neh2 domain and firefly luciferase in human neuroblastoma cell line SH-SY5Y. **Validation studies** (see Smirnova et al 2011) included: (1) Testing of canonical activators of Nrf2, such as 15-deoxy-prostaglandin J2 (15d-PGJ2), sulforaphane and tert-butylhydroquinone (TBHQ), which were shown to produce a significant increase (> 20-fold) in the background luciferase signal within 3-4 h following the treatment of SH-SY5Y human neuroblastoma cells stably expressing the Neh2 reporter; (2) Forced expression of Keap1 in the Neh2-luc reporter cell line led to a 3.5-fold decrease in the background luminescence; (3) Keap1 reduction by siRNA resulted in a steady state increase in Neh2-luc reporter luminescence and an induction of transcription of Nrf2-regulated genes; (4) Immunoblot with antibodies against luciferase confirmed that an

increase in luminescence corresponds to the accumulation of the fusion protein upon incubation with commonly used Nrf2 activators. Validation studies demonstrated that Keap1 regulates the stability of the Neh2-reporter in the same manner as for endogenous Nrf2 and that the rate-limiting step in Neh2-luc cell line is controlled by the disruption of the Neh2-Keap1 complex which results in inhibition of ubiquitinylation. The Neh2-luc reporter system is a novel tool to monitor the direct effect of a particular compound on the first step controlling Nrf2 stability, i.e. Nrf2-Keap1 and/or Keap1-Cul3 interaction.

### 2.1.3 Neh2-luciferase reporter sensitivity

All manual screenings were performed using 96-well plates with 100  $\mu$ L serum per well dispensed with WellMate multichannel dispenser from Matrix (Thermo Fisher Scientific). Drugs were added after overnight culture incubation, 3hr later serum removed, lysis buffer added, and luminescence measured with either BrightGlo reagent (Promega) or common luciferase reagent made by ourselves. The background luminescence signal calibrated with recombinant luciferase allows us to estimate the steady-state concentration of the Neh2-luciferase fusion protein: the background is ca. 15-20 rlu, which corresponds to 0.25-0.33 pg luciferase protein and is more than two orders of magnitude lower than that observed for the cell line expressing wild-type luciferase under control of the same promoter. The low steady state luciferase activity (recalculated as 0.6-0.8 nM fusion protein for 30,000 cell/well density and 233  $\mu$ m<sup>3</sup> single cell volume) suggests that in spite of the forced expression of the Neh2-luciferase fusion protein, it is successfully recognized by the endogenous Keap1-Cul3 complex and almost fully degraded. The reporter exemplifies the action of an “ideal Nrf2 activator” which stabilizes endogenous Nrf2 by competing for Keap1 binding and not by modifying Keap1 chemically. The new reporter is advantageous over the ARE-luc reporter or especially ARE-GFP reporter (Shaw et al 2010) and other methods such as immunoblotting of Nrf2 (or luciferase in our case) or RT-PCR of Nrf2-induced genes because all other methods are at least 5-6 hr delayed as compared to immediate response of the newly constructed reporter. Moreover, ARE-luc reporter background signal is at the single rlu digits and activation for TBHQ is only 3-fold compare to 16-20 fold activation in the case of Neh2-luc reporter (see Smirnova et al 2011).

There are no examples of the literature on the application of the same approach to screening purposes, except for the recently published paper on the reporter construct expressing a fusion between three domains of Nrf2 and beta-galactosidase (Hirotsu et al 2011). The authors validated the construct and showed the advantages of the reporter over the commonly used ARE-luciferase. Nrf2d-LacZ protein consists of Nrf2 N-terminal region (containing Neh2, Neh4, and Neh5) and SV40 nuclear localization signals (NLS)- $\beta$ -galactosidase, it is specific to Nrf2 activators. However, the Nrf2d-LacZ reporter it is still less sensitive than the one developed in our laboratory. Nrf2d-LacZ reporter provides ca. 5-fold activation over the background signal upon addition of a classic Nrf2 activator, TBHQ, and requires at least 1 hr incubation to get the first time-point (see Fig 3C in Hirotsu et al 2011), compared to 20-fold activation for TBHQ (see above) and the possibility to monitor the reporter effect minutes after drug addition.

### 2.1.4 Neh2-luciferase reporter optimization for HTS format

For HTS purposes, the assay was optimized to 384-well format: SH-SY5Y-Neh2-luc cells were plated into 384 well, white, flat-bottom plates at 7000 cell/well in 30  $\mu$ L serum and

incubated overnight at 37°C, 5% CO<sub>2</sub>. The next day compounds were added to two final concentrations of 10 µM and 20 µM, plates were incubated for 3 hr at 37°C, and luciferase activity was measured using SteadyGlo™ reagent (Promega). Each plate had two internal standards, TBHQ (100%) and DMSO (0%). This format provides Z values above 0.7. The reporter activation (%) was calculated as a ratio  $(L - L_{\text{DMSO}}) / (L_{\text{TBHQ}} - L_{\text{DMSO}})$ . Hits were defined as those greater than 25%. The pilot screen of Spectrum library revealed 224 hits exhibiting Neh2-luc reporter activity equal or higher than 25% of TBHQ; among those, 100 showed activation of at least 75% of that induced by TBHQ. Thus, 5% of biologically active compounds and drugs presented in the Spectrum library are at least 75% as potent as TBHQ in activation of Nrf2. The prevalence of hits does not indicate the low specificity of the reporter, it simply reflects the important role that Nrf2 plays in xenobiotic detoxification of a large number of chemical entities. Ideally, any compound disturbing cellular redox balance should be a hit in Neh2-luc screen.

HTS of Spectrum library of 2,000 biologically active and FDA-approved compounds was performed using a control cell line expressing plain luciferase under the same promoter to evaluate the effect of compounds on luciferase activity. None were found to inhibit/enhance the luciferase activity under the experimental conditions, while 46 compounds were found to be toxic at 3 hr incubation and were excluded from consideration. Cell death was monitored simultaneously with luciferase assays by plating cells, in parallel, in the transparent bottom plates and performing two independent assays of cell viability along with luciferase: MTT reduction and phase contrast observation. In all cases, MTT agreed with our morphological assay. The use of robotics for cell plating results in uniform concentration of cells along the plate, and we have found after validation no need to continue normalization to the cell protein. As one might expect, additional manipulations in the same well result in increasing the errors in following activity measurements as we established during the HTS optimization.

Further HTS screens are performed with two reporters in parallel, e.g. Neh2-luc and HIF1 ODD-luc (see 2.2), in this case each reporter serves as a control for the other. Hits that are equally active in both reporters are excluded from further consideration (could be either metal chelators, redox active compounds, or proteasomal inhibitors). Hits that do not overlap are subject to further studies. In the particular case of HTS for non-alkylating Nrf2 activators, hits are passed through the structural filters to remove pro-oxidant, alkylating and other reactive motifs recently described by Baell & Holloway 2010.

#### **2.1.5 Challenge to find specific Nrf2 stabilizers: Advantages of real-time monitoring**

A major challenge in the development of effective Nrf2 activators is to identify those that lead specifically to Nrf2 stabilization and subsequent promoter activation, without imposing general oxidative/electrophilic stress. Such activators should exhibit higher affinity for Keap1 and work by competing with Nrf2 Neh-2 domain for Keap binding. The novel reporter for the first time provides the possibility of real time monitoring for changes in the stability of Nrf2 in the form of the luciferase labeled Neh2 domain. By following the kinetics of reporter activation one may expect to discriminate the mechanism of action of various Nrf2 activators, i.e. direct activators will exert immediate effects, while those acting indirectly will show lag-periods of different durations. Kinetics of reporter activation were measured by adding varied fixed concentrations of an inhibitor at different time points followed by simultaneous cell lysis and activity measurement in the whole 96-well plate;

this assay format minimizes experimental error originating from the well-known instability of a luciferase reagent.

There are 6 types of experimentally observed kinetics of reporter activation (Fig.4).

Type 1 is characteristic for alkylating agents such as sulforaphane, pyrithione, auranofin, etc. and shows an immediate linear increase in reporter signal in a concentration-dependent manner, and eventually reaches a threshold plateau (determined as a background signal magnitude in the control wild-type luciferase line).

Type 2 shows lag-periods that shorten with rising concentrations and eventually disappear pointing to a switch in the rate-limiting step to the one affected by the added compound: this time-course is typical for proteasomal inhibitors and is observed for both Neh2-luc and HIF ODD-luc reporters (see Fig.1 in Smirnova et al, 2011).

Type 3 shows a prolonged lag-period which duration shortens with rising concentrations to a some limit, but never disappears: such time-course indicates that the drug effects on a step with a limited contribution to the rate-limiting step. It has been seen only for cadmium among the activators studied: the duration of a lag-period was decreased to 1 hr, and the subsequent increase in cadmium concentration had no effect.

Type 4 shows prolonged lag-period, which duration is concentration independent. This type is characteristic for drugs working either upstream of Nrf2 stabilization step (such as Hsp90 inhibitors showing 3 hr lag-period), or those drugs which must be initially oxidized to be able to modify Keap1 thiols (catechol with 20 min lag-period, or o-phenylene diamine with 40 min lag-period).

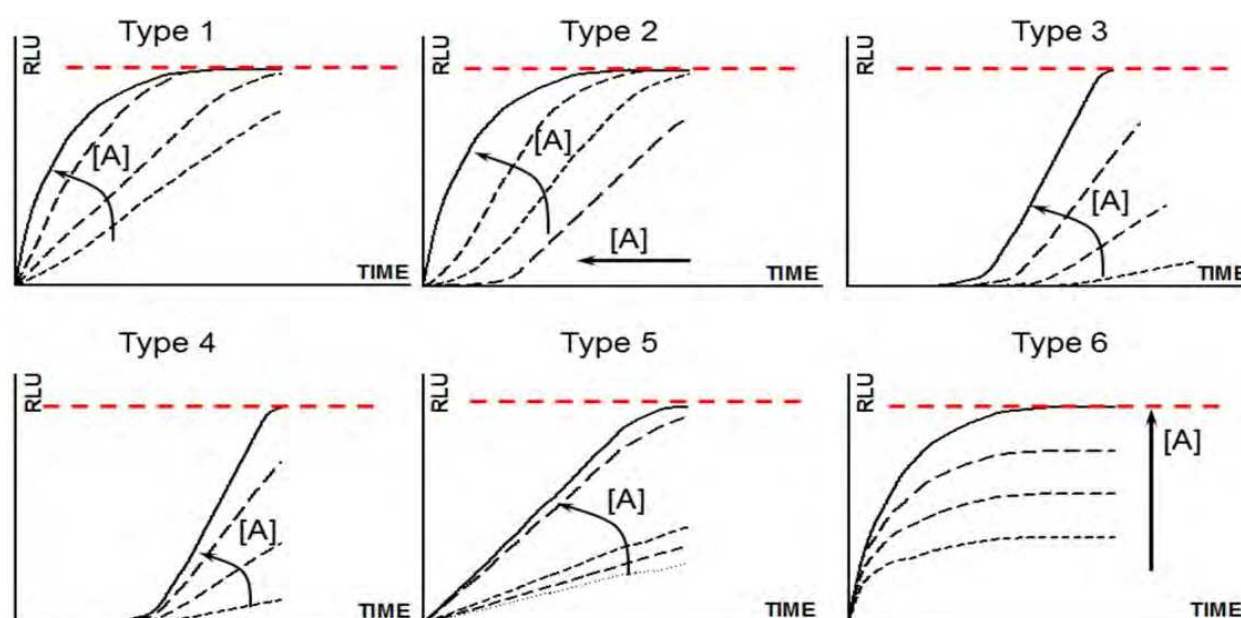


Fig. 4. Schematic representation of various types of reporter activation time-course (see text for explanation). Arrows indicate rising concentrations of Nrf2 activator, [A]; red thresholds show maximum reporter activation determined using a control cell line expressing wild-type luciferase under the same promoter.

Type 5 has been observed for two best hits in Spectrum library, nordihydroguaiaretic acid (NDGA) and fisetin, and shows an immediate switch-type response: there is negligible growth in signal up to a specific concentration (ca. 1.5-2  $\mu$ M) which brings the system to the maximum rate of signal increase. At this point, we can speculate that Keap1 serves as a



redox sensor, possibly via zinc atom bound to some of its thiols. There is evidence for zinc presence *in vitro* for recombinant Keap1, no data available on zinc binding to Keap1 *in vivo*. Future studies are necessary to clarify the target for these two compounds, which were confirmed to be extremely potent Nrf2 stabilizers (Smirnova et al 2011).

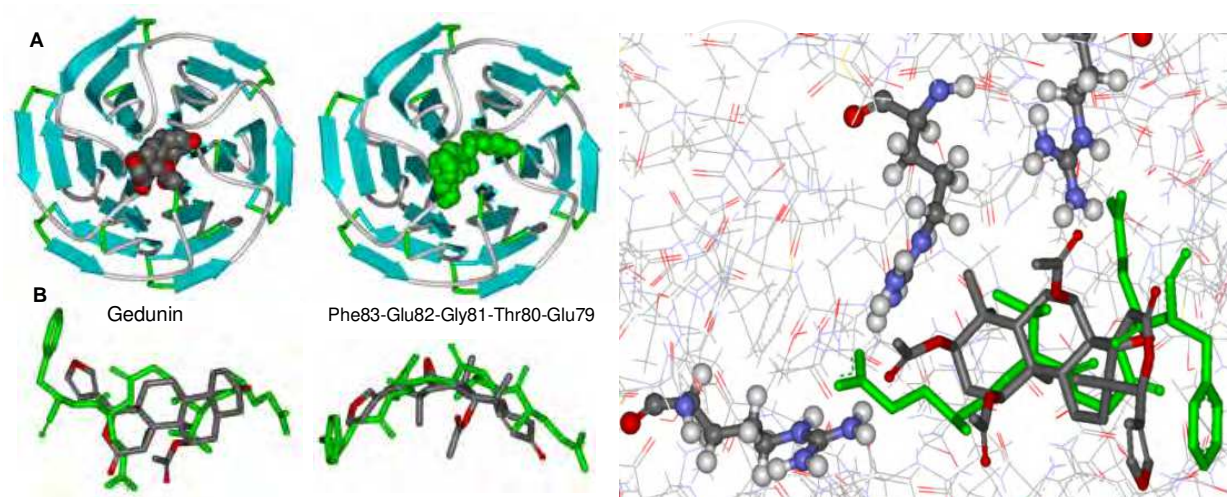


Fig. 5. Gedunin (Left: A & B) and 7-deacetoxy-7oxokhivorin (Right) docking into Keap1 in comparison with the corresponding portion of Neh2 16-mer peptide (green). **Docking experiments** are performed using the CDOCKER algorithm, followed by force field minimization and binding energy calculations using the molecular mechanics algorithm CHARMM (as implemented in Discovery Studio 2.5, Accelrys, San Diego, CA). The crystal structure of the human Keap1 kelch domain with the bound 16-mer peptide of human Neh2 (2FLU.pdb) with polar hydrogen atoms added was used as the starting template structure.

Type 6 is the one that is most promising in terms of specific Nrf2 stabilization: a time-course with a concentration dependent re-equilibration plateau is usually a characteristic of competitive binding. This type of kinetics has been observed for gedunin and some of its derivatives. Gedunin fits perfectly into the same Keap1 binding pocket as Nrf2 closely following the bending of the 83FEGTE79 portion of Nrf2 peptide (Fig. 5 left). Moreover, its oxykhivorin analog with Michael's motif removed and replaced with two acetoxy-groups fits just perfectly, and is a more powerful activator of the Neh2-reporter than gedunin itself (Fig.5 right).

We consider targeting the site of Neh2 binding in Keap1 as the most promising approach for the development of Nrf2 activators not imposing general oxidative stress. Another approach has been recently proposed by Wu et al (2010) who constructed a model of Keap1 including intervening region. Targeting this region one may expect to disturb Keap1 conformation in such a way that Nrf2 is released from the complex. This approach in general is less specific since Nrf2 is not the one client of Keap1.

The authors performed virtual screening using their model and identified a number of structures showing decent docking scores (Fig. 6). Experimental testing of the identified compounds showed that only B10, B31, and B40 are good inducers of Nrf2-dependent genes. The confirmed hits are redox-active molecules and their effect on Keap1-Nrf2 interaction may be not specific.

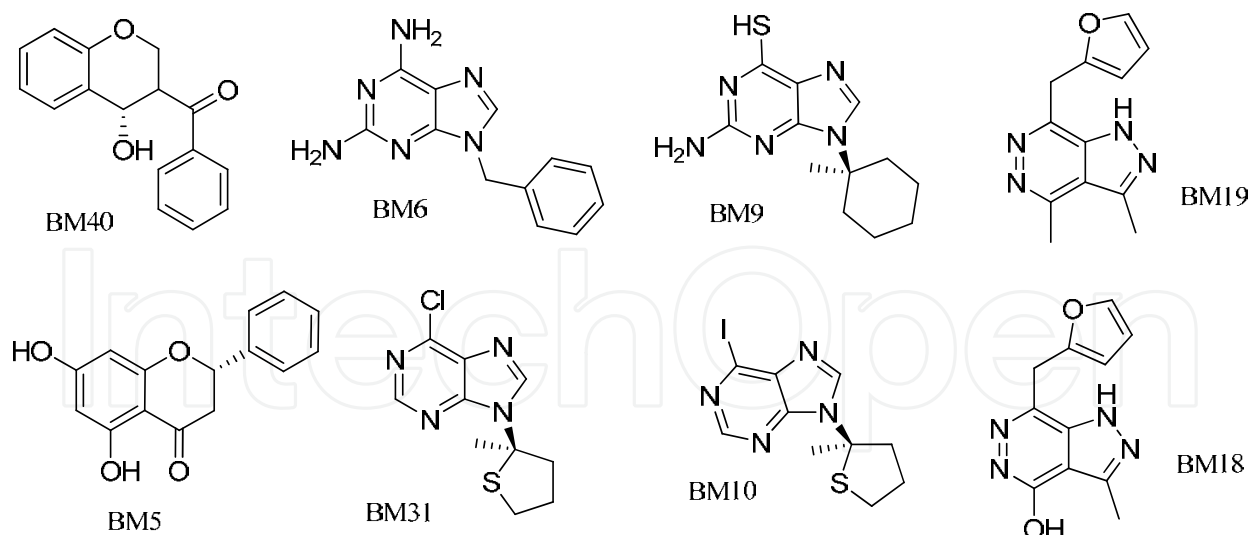


Fig. 6. Virtual hits targeting intervening region of Keap1 (Wu et al 2010).

#### 2.1.6 Criteria for selection of compounds for hit-to-lead program

Depending on a particular goal, an algorithm for drug development efforts will involve a number of steps confirming the nature of the drug action mechanism. For example, if our goal is to develop Nrf2 specific activators that work specifically via disruption of Nrf2-Keap1 interaction by competitively displacing the Neh2-portion of Nrf2, and not by non-specific alkylation of Keap1 thiols, we must perform biological analysis to confirm this. The detailed structure-activity relationship studies (SAR) will employ (a) concentration titration experiments; (b) time-course of reporter activation; (c) *in silico* modeling; the hits obtained from screening the library will be used to help refine the *in silico* model; (d) Keap1 labeling experiments in the presence of selected hits to prove the non-alkylating nature of activation (see Smirnova et al 2011 Fig. S5A & B and protocol therein); (e) hits testing for the ability to upregulate Nrf2-dependent genes (RT-PCR); (f) evaluation of toxicity using primary culture neurons; (g) hits that upregulate Nrf2 may be analyzed by microarray for non-specific activation of Nrf2-independent pathways; (h) hits that are neuroprotective *in vitro* are tested for ADME (Absorption, Distribution, Metabolism and Elimination).

We have chosen two of our hits from Spectrum library – NDGA and gedunin for hit-to-lead program which is currently in progress. Both compounds are the key components of well known herbal medicines used for centuries by Native Americans (chapparal) and Indians (neem tree), respectively. For both compounds, ADME studies have been already done, and the compounds are neuroprotective in the *in vivo* model of MPTP-induced toxicity.

*In vitro* ADME studies have always played a critical role in optimizing the pharmacokinetics (PK) properties of lead compounds thereby increasing their success rate (Thompson 2000). The ideal PK properties for an oral drug are favorable bioavailability, clearance and metabolic stability to provide adequate systemic exposure to elicit a pharmacodynamic response with low potential for dose-dependent toxicities. These *in vivo* properties should be assessed as early as practical using *in vitro* ADME prediction tools (Balani et al 2005). In 1991, PK properties were attributed to more failure of drugs (40%) than efficacy or safety in clinical trials. With the application of advanced ADME techniques, the contribution of PK properties resulting in drug failure dropped dramatically to 10% in 2000 (Kola & Landis 2004). Recently, *in vitro* ADME and cytotoxicity screening assays have been incorporated

with structure-activity relationship (SAR) studies in the earliest stages of drug discovery. The incorporation of early, high throughput ADME screening in parallel with efficacy screening has significantly reduced cycle time involved in moving compounds from “hit” to “lead” status.

## 2.2 Cell-based screening using HIF1 ODD-luc reporter system

Major advantage of cell-based screens is that we automatically exclude non-permeable and toxic compounds because they provide either no activation or even lower the background signal. In addition, there is no need to specifically formulate the composition of a reaction medium for an enzymatic reaction because the intracellular medium of cell lines is very close to that existing in the human body. Another advantage is that compared to *in vitro* enzymatic assay we rule out enzyme stability problem. The best example for this is the reaction catalyzed by HIF prolyl hydroxylase (HIF PHD). Below we present a detailed comparison of enzymatic screens versus cell-based screen for HIF1 activators working via inhibition of HIF PHD.

### 2.2.1 HIF and HIF prolyl hydroxylase: HTS assay development problems

Hypoxia-inducible factor (HIF) is a transcriptional factor that regulates gene expression in mammalian development, physiology and disease pathogenesis (Wang et al 1995). HIF consists of an oxygen-sensitive  $\alpha$ -subunit and a continuously expressed  $\beta$ -subunit. HIF-1 $\alpha$  is induced and stabilized in hypoxic conditions and functions as a master regulator of oxygen homeostasis. HIF-1 $\alpha$  proteolysis is mediated via hydroxylation of two highly conserved Pro residues, which causes its direct interaction with von Hippel-Lindau protein (VHL). HIF-1 $\alpha$  hydroxylation is catalyzed by human prolyl-4-hydroxylases (PHDs). Pro564 is located in the so called oxygen degradable domain (ODD) and is considered as the major site for hydroxylation catalyzed by all PHD isoforms. For HIF PDHs, the general reaction mechanism (Solomon et al 2000) includes activation of molecular oxygen to convert  $\alpha$ -ketoglutarate into succinate and CO<sub>2</sub> while hydroxylating the prime substrate, HIF- $\alpha$  subunit (Kaelin 2005). The reaction proceeds via the formation of oxoferryl-intermediate as shown in Fig.7.

The proposed mechanism is based on the reported incorporation of <sup>18</sup>O into HIF- $\alpha$  substrate and succinate, the available crystal structures for PHD2, and the recent stopped-flow kinetics for taurine/ $\alpha$ -ketoglutarate dioxygenase and prolyl-4-hydroxylase from *Paramecium bursaria Chlorella virus 1* (Price et al 2005). The mechanism suggests the initial binding of iron to the active site, then  $\alpha$ -ketoglutarate coordination via C-1 carboxylate and ketone oxygen by iron, followed by the binding of HIF peptide (as a substrate) which results in displacing the water molecule from the 6<sup>th</sup> coordination position. This displacement guarantees oxygen binding and activation. The uncoordinated oxygen of the bound oxygen attacks the ketone carbonyl of  $\alpha$ -ketoglutarate to form a bicyclic Fe(IV)-peroxyhemiketal complex, in which decarboxylation occurs concomitantly with formation of an oxo-ferryl (Fe(IV)=O) intermediate. The latter one hydroxylates proline via a substrate radical intermediate as evidenced by the formation of prolyl radical: i.e. oxo-ferryl attacks proline residue to withdraw hydrogen atom, and then introduces the hydroxyl radical. The presence of the water molecule in the 6<sup>th</sup> position does not prevent oxygen from binding and activation completely: in the absence of the substrate the enzymes of this class are known to catalyze the so called uncoupled reaction, i.e. ketoglutarate decarboxylation in the absence of HIF or HIF peptide. However, the rate of the reaction is rather slow.



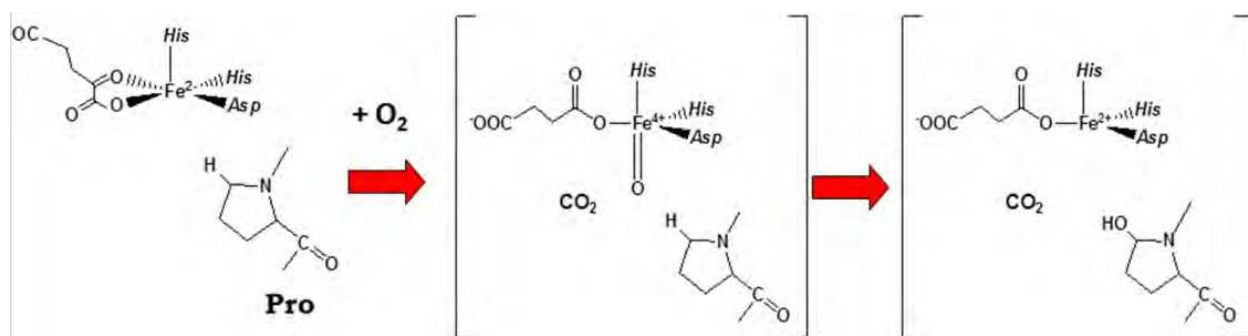


Fig. 7. Schematic presentation of two half-reactions of HIF prolyl hydroxylase catalytic cycle (see detailed explanation below). The reaction substrates are ketoglutarate, oxygen and HIF or HIF peptide, and the reaction products are succinate, carbon dioxide, and hydroxylated HIF or HIF peptide.

PHDs 1-3 (also known as EGLN 2,1, and 3) in human are represented by three isozymes with catalytic domains very homologous to each other (Bruick & McKnight 2001). PHD3 contains the catalytic domain only, contrary to PHD1 and PHD2, which have additional N-terminal domains. The crystal structure of PHD2 catalytic domain was resolved by two independent groups. PHD2 exists as a monomer in solution. The enzyme contains double-stranded  $\beta$ -helix core fold common to the Fe(II)- $\alpha$ KG-dependent dioxygenase family. The active site comprises a relatively deep pocket compared to other  $\alpha$ KG oxygenases. Iron is coordinated in an octahedral manner by His-313, His-374 and Asp-313, inhibitor (occupying two sites) and water molecule (Fig.8). The similarity in the structure of catalytic domains of PHDs1-3 and identical  $\alpha$ KG binding residues (according to the homology modeling) makes the task of developing specific inhibitors based on  $\alpha$ KG analogues doubtful.

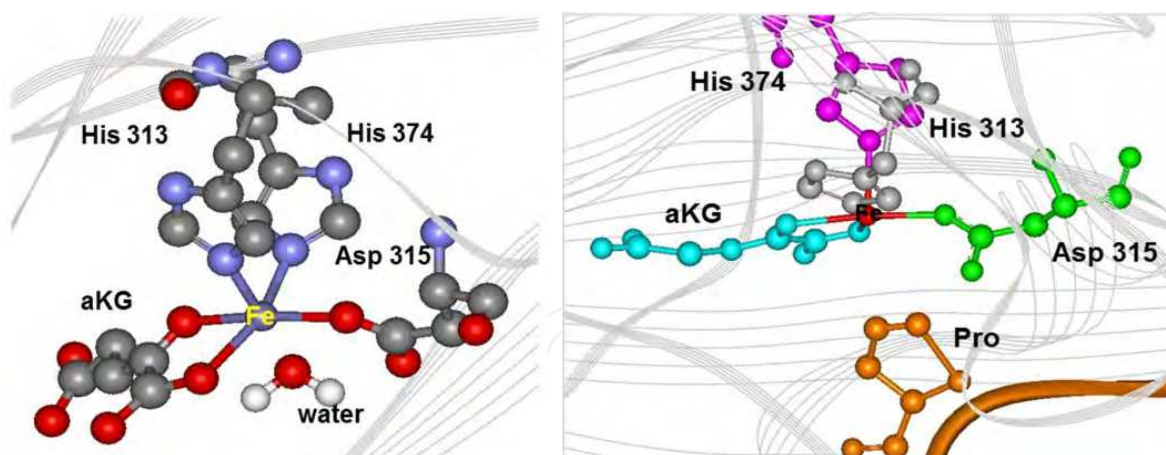


Fig. 8. Displacement of active site water (left) upon binding of HIF peptide (right) initiates oxygen binding and the catalytic cycle of ketoglutarate decarboxylation accompanied by formation of ferryl iron, subtraction of hydrogen from Proline 564, and subsequent hydroxylation of the latter.

There are 3 different ways to assay PHD activity (Hewitson et al 2007):

- The assay of the first half-reaction substrates and products (O<sub>2</sub> and a-ketoglutarate consumption; CO<sub>2</sub> evolution, succinate production); this format should account for the uncoupled reaction, which is non-specific.



- The assay of the second half-reaction oxidized product (mass-spec of hydroxylated peptides); mass-spec analysis is difficult to make suitable for HTS format.
- The so-called “capture assay” monitoring interaction of the hydroxylated product with VHL; requires recombinant HIF, reticulocyte-produced VHL, protein/peptide labelling and corresponding antibodies.

The most reliable way to monitor the formation of hydroxylated product is either by mass-spectrometry, or using the “capture assay”. The latter is known in three different formats:

- **End-point** immunochemical assay is based upon the tight interaction between pVHL and the hydroxylated Pro564 of HIF-1 $\alpha$ . Immobilized HIF-peptide which, after hydroxylation, is recognized by the thioredoxin-labeled VHL-elongin B-elongin C complex (expressed in *E.coli* and purified), which is its turn, is detected by anti-thioredoxin antibodies by the method of double antibodies with the second, peroxidase-labeled, antibodies.
- **Continuous** fluorescence polarization assay: HIF-peptide modified with a fluorescent label upon capture by VHL-elongin B-elongin C shows higher polarization signal (Cho et al 2005).
- **Continuous** homogeneous time-resolved fluorescence resonance energy transfer (TR-FRET) assay: VCB-Eu complex recognizes hydroxylated P564-HIF-1 $\alpha$  peptide, preliminary biotinylated, followed by interaction with streptavidin labeled allophycocyanin (Fig.9). This assay, developed by Amgen team, is the best one in terms of sensitivity and applicability for HTS. The assay, in addition to other expensive reagents, utilizes Europium (Dao et al 2009).

All enzymatic assays are based on the use of PHD2 recombinant enzyme produced in either baculovirus or *E.coli* expression system. High throughput screening for PHD inhibitors using an enzyme assay is a challenge both in terms of the enzyme source and the assay format. The enzymatic activity and stability of purified PHD is very low (Tuckerman et al 2004), and enzyme assays suitable for HTS require large quantities of recombinant enzyme supplemented with iron. One of challenges in the search for selective HIF PHD inhibitors or other regulators of HIF stability is to discriminate between non-specific iron chelation in solution and specific iron chelation inside the active center of the PHD enzyme (see 2.2.4). Given the non-physiological conditions under which screening for inhibitors occurs with recombinant PHD2, it is not surprising that the IC<sub>50</sub> value determined in the enzyme *in vitro* assay did not correlate with the IC<sub>50</sub> for VEGF activation reported in the same study (Warshakoon et al. 2006d). Another limitation is the use of a 19-mer HIF peptide, whose affinity for the HIF PHDs is orders of magnitude lesser than the full length protein. So far only Amgen team used recombinant HIF protein in HTS of their internal collection, although again they had been varying the concentration of  $\alpha$ KG, not HIF, when determining the inhibition constant for their best hit (Tegley *et al.* 2008). A negative consequence of the test tube strategy is the assay format is more likely to yield inhibitors competitive with respect to  $\alpha$ KG than those competing with HIF itself.

The screening for PHD2 inhibitors based on enzymatic assays with recombinant PHD2 was performed by Fibrogen, Procter&Gamble, Amgen, and some other groups. The Procter&Gamble team published 4 papers analyzing structure-activity relationship (SAR) for 3 different groups of compounds possessing clear iron binding motifs that dock into the PHD2 active site. The hydroxylation assay was performed by mass-spectrometry. In addition, the ability of some inhibitors to induce VEGF was judged by a VEGF ELISA using

HEK293 cells. The total number of compounds reported was ca.50. The screening for PHD inhibitors based on the  $\alpha$ KG mimetic structures yielded a number of different classes of inhibitors with the inhibition constants in the  $\mu$ M range. The best inhibition constant among substituted pyridine derivatives was exhibited by *p*-chloro phenyl-substituted 5-pyridine carboxamide (15  $\mu$ M) and those with the pyrazole moiety in the 5<sup>th</sup> position of the pyridine ring (5-20  $\mu$ M) (Warshakoon et al, 2006a); the further work resulted in the significantly improved affinity (1-2  $\mu$ M) of the newly designed inhibitors of pyrazolopyrimidines series (Warshakoon et al, 2006a). Imidazo[1,2-a]pyridine derivatives showed the apparent inhibition constants in the 4-27  $\mu$ M range (Warshakoon et al, 2006b), and 8-hydroxyquinoline derivatives exhibited the apparent inhibition constants in the range of 3-10  $\mu$ M (Warshakoon et al, 2006c). However, taking into account that the screening for potential inhibitors was performed using a 19-mer peptide, which shows at least 1 order of magnitude worse  $K_m$  than HIF-ODD, the range of the apparent inhibitor constants obtained may be insufficient for the inhibition of the enzyme activity *in vivo*.

Very recently, the Amgen team developed a time-resolved fluorescence resonance energy transfer-based assay based on interaction of the hydroxylated HIF peptide with VHL and performed HTS of their "internal compounds collection" to reveal a lead compound of IC<sub>50</sub> of ca. 72 nM from a novel hydroxyl-thiazole class, followed by lead optimization. 30 new derivatives have been synthesized and assayed. The inhibition constant was determined from single-point measurements done in triplicate at pH 6.0, which was found to be optimal for the *in vitro* reaction catalyzed by human full-size recombinant PHD2. The best inhibitors have a long hydrophobic tail that screens the entrance to the active site. Flexibility of the tail (benzyl instead of phenyl) enhances affinity by orders of magnitude as the IC<sub>50</sub> is improved from dozens nM to single digits. These are the best inhibitors reported in literature thus far.

The conformation of recombinant PHD2 may be different from that of the native enzyme, in addition, PHD2 like all enzymes of this family is highly unstable in the absence of reducing agents such as DTT. The recently reported crystal structure of PHD2 with a 17-mer HIF peptide (Chowdhury *et al.* 2009) shows no active site water displacement, which appears to be a mandatory requirement for the initiation of the catalytic cycle. Given these biases, it is not surprising that all PHD inhibitors developed using the recombinant enzyme explored only the  $\alpha$ KG-binding motif inside PHD2 active site and had a carboxyl group interacting with Arg-383 in addition to a clearly defined iron-binding motif (Tegley et al. 2008, Warshakoon et al. 2006a, Warshakoon et al. 2006b, Warshakoon et al. 2006c, Warshakoon et al. 2006d, Ivan et al. 2002). The reaction mixture has to be supplemented with excess of iron, alpha-ketoglutarate, and HIF peptide, making the selection of mild inhibitors impossible. Majority of PHD inhibitors identified in HTS are presented by iron chelators. The only exception so far is Compound A with IC<sub>50</sub>=0.032  $\mu$ M (see Fig.9 right) identified by Amgen team using the newly developed assay (Fig.9), however, the core motif will not pass the filters recently described by Baell and Holloway, 2010.

One of disadvantages of enzymatic assay is that despite the apparent specificity, the hits identified in HTS may potentially target other enzymes of the same class. In particular, human genome has more than 70 genes of putative non-heme iron dioxygenases, and there is no principal difference in organization of the active sites of these enzymes. As a rule, the major difference is in the access to the active sites. There are three different forms of HIF (1-3) and three PHD isoforms, and all data reported so far on enzyme specificity using HIF

peptides and recombinant enzymes give no clue on the preference for a particular substrate for a PHD isoform. On the other hand, the study of HIF-PHD interaction in yeast two hybrid system indicated principal differences existing among PHD isoforms with respect to recognition of HIF isoforms (Landazuri et al 2006). The latter observations are in agreement with our results obtained with HIF2 ODD-luc and HIF3 ODD-luc reporters showing that HIF3 is not recognized by PHD1 and PHD2. Therefore, running enzymatic assays with PHD2 and HIF peptides does not give a true picture of enzyme specificity and cannot be used for development of isozyme-specific inhibitors.

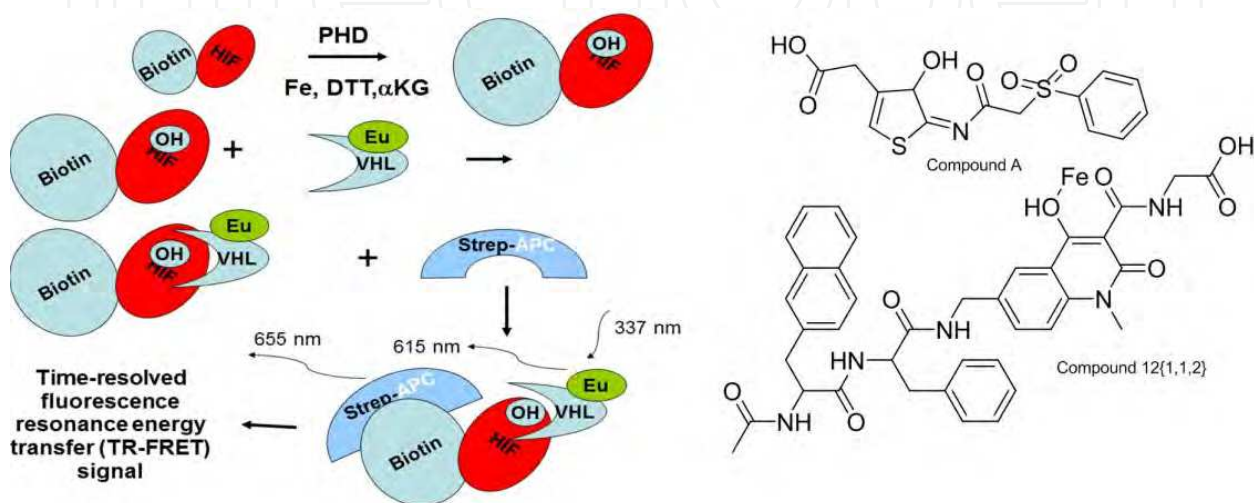


Fig. 9. Schematic presentation of TR-FRET assay (left) developed by Amgen team to monitor the activity of HIF prolyl hydroxylase. The assay employs biotin-labeled HIF, Eu-labeled VHL protein, as well as APC-labeled streptavidin, in addition to the recombinant PHD2 used as a catalyst of the first enzymatic step. The assay was successfully used for HTS purposes: an inhibitor (E)-2-(4-hydroxy-5-(2-(phenylsulfonyl)acetyl)imino)-4,5-dihydrothiophen-3-yl)acetic acid specific for αKG binding site has been developed (right, Compound A), and later dipeptidyl-quinolones (see compound 12{1,1,2}) specific to PHD1 and 3, and 10-fold less specific to PHD2 (Murray et al 2010).

### 2.2.2 HIF1 ODD-luciferase reporter construction (Safran et al 2006, Smirnova et al 2010)

Two primary modes of screening for HIF activators have been well described: a recombinant enzyme-based screen for PHD2 inhibitors (used by Fibrogen (Ivan *et al.* 2002), Amgen (Tegley *et al.* 2008, Allen *et al.* 2008)), Procter and Gamble Pharmaceuticals Inc. (Warshakoon *et al.* 2006a, Warshakoon *et al.* 2006b, Warshakoon *et al.* 2006c, Warshakoon *et al.* 2006d), and other teams (Nangaku *et al.* 2007)); and a cell-based screen using HRE-luciferase reporter construct used by a number of labs including our own.

The cell-based assay with HRE-luc reporter system, a promoter-reporter construct that contained 68 bp of a known hypoxia and HIF-1 regulated gene, enolase, containing a wild type HRE (5'-RCTGT-3'), is a widely used approach for screening of HIF activators with diverse mechanisms of action (Semenza *et al.* 1996). A reporter system is based on transfected immortalized hippocampal neuroblast cell line (HT22) and allows screening for a broad spectrum of compounds that include: activators of HIF transcription; activators of HIF binding to HRE; and effectors of HIF protein stability (PHD inhibitors, pVHL &

proteasome inhibitors). The manual screen of Spectrum library performed in this laboratory using HRE-luc/HT22 line took half a year and resulted in 43 hits. However, in our hands, the cell line's response to positive controls decreases after 7 passages, making the system not suitable for a robotic HTS on 384-well plates.

Taking into account the low specific activity of recombinant enzymes, and the inadequacy of interpretation of the inhibition constant generated using different types of enzyme *in vitro* assays, we developed a cell-based reporter system for HTS of ODD-luc stability, a variant of the cell-based "capture" assay, and accomplished a screen of 85,000 structurally diverse compounds (Smirnova et al 2010). Cell-based assay for HIF1 activators working via inhibition of HIF PHDs employs the same strategy as the one used for screening of Nrf2 activators. The HIF1 ODD-luc reporter system is equivalent to *in vivo* capture assay monitored by consumption of a labeled substrate (ODD-luciferase) (see Fig. 10 left). The reporter system II (Fig. 10 right) consists of the HIF-1 $\alpha$  gene fragment encoding the oxygen degradable domain (ODD) containing the key proline residue followed by luciferase gene (*luc*) (Safran et al 2006). The regulation of luciferase protein stability in this reporter system is the same as the physiological activation of HIF: hydroxylation of oxygen-degradable domain (ODD, which contains 530-653 a.a. of HIF1 $\alpha$ ) results in recognition of the ODD-luc fusion protein by VHL followed by its ubiquitinylation and proteasomal degradation (Fig.10), and as we present below, the approach proved to be productive for HTS purposes.

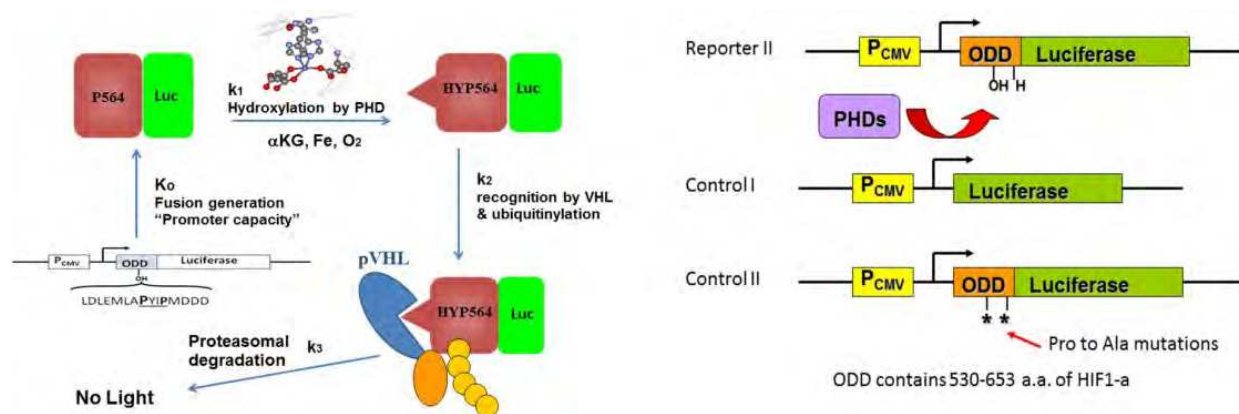


Fig. 10. The principle of HIF1 ODD-luc reporter performance (left) and construction of the two controls (right).

The reporter cell lines constitutively expressing HIF1 ODD-luc (human neuroblastoma, SH-SY5Y) were stable for more than 1 year without significant change in their response to canonical PHD inhibitors such as DFO, dihydroxybenzoate, dimethyloxalylglycine, and ciclopirox (see Smirnova et al 2010, Fig.1B). In order to verify the specificity of luciferase changes as an assay for PHD activity, several control lines were developed: the control line I expressing wild-type luciferase under the control of P<sub>CMV</sub>-promoter, and the control line II expressing ODD-luc with proline 564 and 567 Mutated to Ala, which generates luciferase fusion that cannot be degraded. Both controls experimentally identify a threshold level of ODD-luc protein attainable in these cells. The background signal for the wild-type HIF ODD-luc line (PYIP) corresponds to approximately 5-6% of the ODD-luc levels in the control lines with wild-type luciferase and AYIA line (double mutant P564A/P567A line) (Fig.10 right). Treatment with 10  $\mu$ M ciclopirox results in a 10-fold increase of a background signal for the ODD-luc reporter (PYIP line), i.e. reaches almost 50% of the threshold value (see



Smirnova et al 2010, Fig.2). These particular conditions are ideal for HTS as they promote the selection of both weaker and more potent inhibitors than ciclopirox.

### 2.2.3 Determination of inhibition constants from kinetics of HIF1 ODD-luciferase reporter activation

In accord with validation studies, the HIF ODD-luciferase reporter system is controlled by the rate of PHD-catalyzed reaction: the response of the ODD-luc reporter to canonical HIF PHD inhibitors, and the increased stability of single-point mutant reporters in accord with the predictions (see Smirnova et al 2010), provided confidence that this system could be utilized for screening for novel small molecule HIF PHD inhibitors. From an enzyme kinetics point of view, the HIF ODD-luciferase reporter system is a “capture assay” monitored by the consumption of a substrate, the heterologously expressed HIF ODD-luciferase fusion protein. As we show below, the reporter activation kinetics can be used to determine an apparent inhibition constant for a particular compound. In the kinetic regime, i.e. monitoring the time-course of luminescence changes (Fig.11), the ODD-luc reporter system permits quantitative characterization of promoter capacity ( $K_0$ , rate of fusion protein generation), enzyme activity, and inhibition constant determination. The rate of fusion accumulation equals to the rate of its production ( $K_0$ ) minus the rate of rate-limiting step, controlled by HIF PHDs, which obeys Michaelis-Menten kinetics, as follows:

$$v = d[\text{ODDluc}] / dt = K_0 - k_1[\text{PHD}][\text{ODDluc}] / \{K_m(1 + [I] / K_i) + [\text{ODDluc}]\} \quad (1)$$

where  $K_m$  is the inhibition constant for a competitive inhibitor,  $k_1$  is rate coefficient,  $[\text{PHD}]$  and  $[\text{ODD-luc}]$  are the concentrations of the enzyme and substrate, respectively. The background luminescence signal calibrated with recombinant luciferase allows us to estimate the steady-state concentration of the ODD-luc fusion protein. Under the conditions used the steady-state value of 60 rlu (relative light units) corresponds to 1 pg luciferase protein; dividing this number by the total cell volume taken as a single cell volume ( $233 \mu^3$ ) multiplied by 30,000 cells/well density (number of cells in a 96 well dish), we get the ODD-luc fusion protein steady-state concentration equal to 2.3 nM, which is way below all reported  $K_m$  values for HIF1 (Ivan et al 2002, Tuckerman et al 2004, Koivunen et al 2006). Therefore, we work under non-saturating conditions with respect to HIF substrate, i.e. optimal conditions for selecting inhibitors competitive against HIF substrate. Moreover, as compared to the *in vitro* assay, which uses a 19 amino acid peptide fragment surrounding the oxygen dependent domain (ODD), our ODD-luciferase construct contains 123 amino acid acids, and thus more closely emulates the behavior of native HIF. We can consider the initial concentration of fusion much lower than  $K_m$  and ignore it in the rate equation:

$$v = d[\text{ODDluc}] / dt = K_0 - k_1[\text{PHD}][\text{ODDluc}] / K_m(1 + [I] / K_i) \quad (2)$$

The capacity of promoter,  $K_0$  can be determined under the conditions of total inhibition of PHD activity by means of complete iron deprivation achieved in the presence of high concentrations of ciclopirox, i.e. when the increase in the ciclopirox concentrations give no further increase in the rate of luciferase signal growth (Fig.11). The intracellular enzyme activity ( $k_1[\text{PHD}]/K_m$ ) can be also determined by dividing the rate of fusion protein accumulation by the steady-state concentration of the fusion protein determined directly from one and the same experiment in luciferase units, without recalculation for the cellular

volume, and corresponds to  $0.05 \text{ min}^{-1}$ . The linear plot of  $1/(K_0 - v)$  versus the inhibitor concentration gives the value of the apparent inhibition constant as the intercept on X-axis (Fig.12):

$$1 / (K_0 - v) = K_m(1 + [I] / K_i) / k_1[PHD][ODDluc]_0 \quad (\text{Eq.3})$$

The apparent inhibition constants determined as an intercept on X-axis for compound 7 and DMOG are 0.0012 mM and 1.3 mM, respectively (see Fig.12), which is in agreement with previous observations on DMOG biological effects exerted in the millimolar range and  $IC_{50}$  reported for PHD2 in vitro assay.

The developed approach is good only if the activation effect is significant, at least 3-fold and higher over the background luminescence, otherwise the experimental error of kinetic measurements is too high to perform the above described calculations.

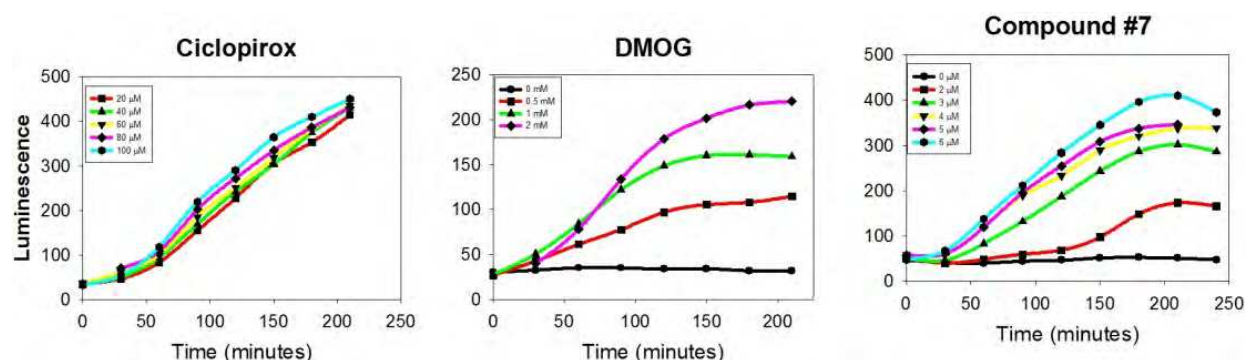


Fig. 11. Experimental kinetics of reporter activation for ciclopirox (complete iron deprivation, which permits determination of the promoter capacity,  $K_0$ ), dimethyloxalylglycine (DMOG), ketoglutarate analog competing with the latter for the binding in the active center of PHD, and one of the best hits in HTS (Smirnova et al, 2010), compound 7 (formula in Fig.12).

#### 2.2.4 Rationale for discriminating between specific and non-specific PHD inhibition

Since the enzyme controlling the rate-limiting step of reporter activation is iron dependent, any iron chelator will come up as a hit in HTS. To discriminate between the non-specific iron chelation in solution and iron coordination by a chelator in the active site of PHD2 we had to develop a rationale. One of the possibilities to evaluate specificity of the reporter response could be the comparison of concentration titration curves in the presence and absence of extra iron. Only specific PHD inhibitors will act at concentrations lower than that of the added iron. Although this approach may potentially yield specific PHD inhibitors right away, it creates a serious experimental problem: cell lines are extremely sensitive to the presence of more than  $2 \mu\text{M}$  extra iron in the medium, which possibly catalyzes Fenton reaction resulting in cell death within hours of incubation. Another approach is to compare reporter activation parameters with the iron chelation ability in solution for a set of compounds of similar structure. If the iron chelation ability of the inhibitors is linearly proportional to HIF activation parameters, it can be safely concluded that the inhibitors simply inactivate PHDs through their ability to chelate iron non-specifically. If on the other hand activation of HIF and iron chelation ability are not proportional then the inhibitors act on PHD through a more specific mechanism.

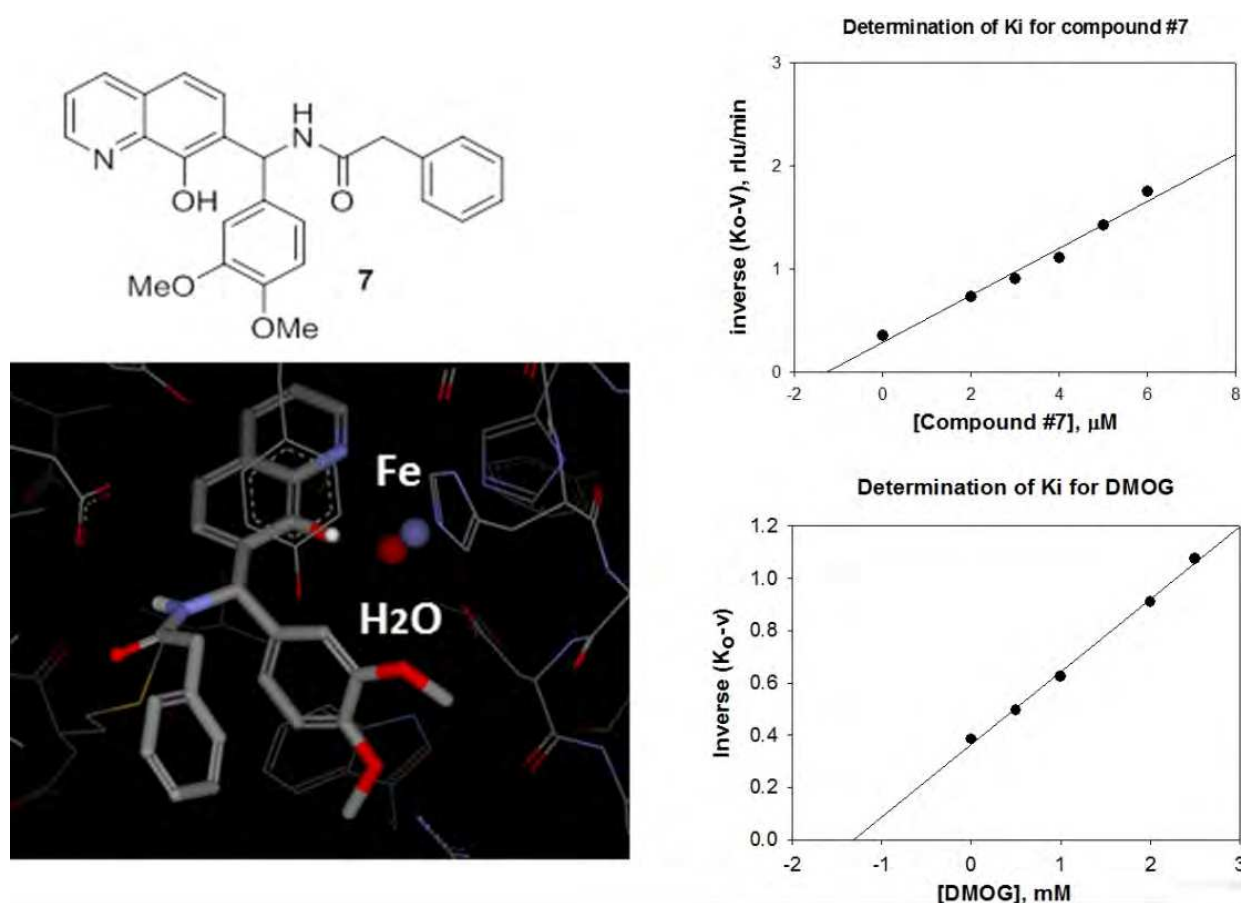


Fig. 12. Calculation of apparent inhibition constants for compound 7 and DMOG using a modified Dixon plot.

To account for iron chelation in solution one should determine the iron binding constant in a model system, for example by displacement of calcein from its complex with iron upon addition of a particular compound monitored by fluorescence (excitation 490 nm, emission 523 nm, cut-off 515 nm) on a Spectramax M5<sup>e</sup> platereader (Molecular Devices). To linearize the fitting equation we introduce parameter  $Y$  as the ratio of calcein-iron complex to free calcein, which can be determined directly from our experiments:  $Y = [Fe-Calcein]/[Calcein]$ . The apparent binding constant for calcein (ca.50 nM) was determined from Fe titration curve for 1 μM calcein in 5 mM Tris-HCl buffer, pH 7.5 (Fig.13 left). The ratio between the iron binding constant for calcein and a particular compound  $K_Q/K_{Ca}$  can be estimated by fitting the titration curve into the dependence of  $[Fe]_o$  vs.  $Y$ , where  $Y = [Ca-Fe]/[Ca]$  is a ratio of calcein-bound Fe to free (fluorescent) calcein:

$$[Fe]_o = K_{Ca}Y + [Ca]_oY / (Y + 1) + [Q]_oY / (Y + K_Q / K_{Ca}) \quad (4)$$

The protocol included addition of 50 μL of Iron in various concentrations 0.5 μM – 2.0 μM to the buffer solution, then the addition of 50 μL calcein so that final concentration of calcein is 1 μM; the addition of 50 μL compound of interest in various concentrations, and then taking end point readings at 20 min incubation. Range of compound concentration was determined through how well the compound liberated calcein from its complex with iron (Fig.13 right). Besides the equilibrium constant, the association rate constant (kinetic coefficient) can be determined as the second order rate constant for calcein displacement kinetics from its

complex with iron (1  $\mu\text{M}$ :1  $\mu\text{M}$ ) upon addition of an oxyquinoline (5-20  $\mu\text{M}$ ), i.e. calculated from the slope of a linear plot of the initial rate of calcein release *vs.* the concentration of oxyquinoline added. This parameter can be used as a kinetic characteristic of iron binding. Once the iron binding constants are determined, one can plot the reporter activation parameters versus inverse inhibition constant, or iron association constant. In both cases,  $\text{IC}_{50}$  determined from the concentration titration curve is the correct parameter to plot (Fig. 14). The activation magnitude is not a perfect parameter for structure-activity relationship (SAR) studies, because as we wrote earlier, there is a threshold for reporter activation, and if incubation is not short, the reporter hits the plateau, which is one and the same for all hits.

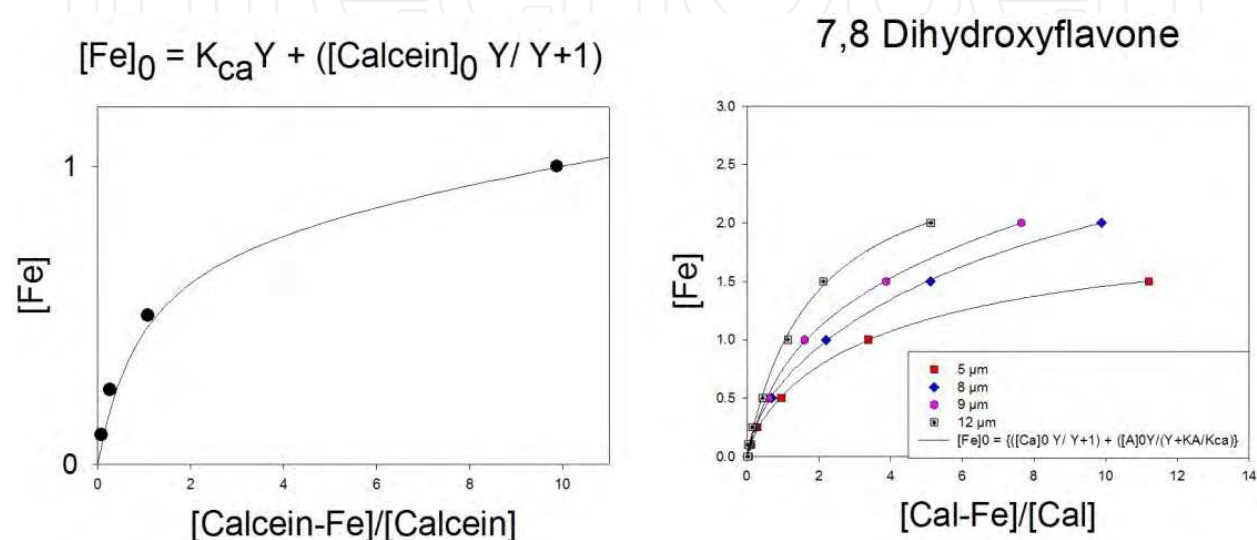


Fig. 13. Determination of iron binding constant for calcein (left) and 7,8 dihydroflavone (right) as an example of iron-binding compound.

As seen in Fig. 14, there is a parallel in iron chelation properties and  $\text{IC}_{50}$  for majority of branched oxyquinolines studied, except for 4 compounds which deviate from the linear dependence (##1,4,7,8). All these have a specific branch similar to that shown in Fig. 12 for compound 7. Compounds 7 and 8 were taken for biological analysis along with compound 10, which was used as a negative control, because despite its iron binding capacity and cell permeability is as good as for compounds 7 and 8, this compound is not a specific PHD inhibitor.

A similar approach can be used to rule out the effect of reducing or oxidative properties (say flavonoids). For this purpose, the rate constant for oxidation/reduction of ferro/ferricyanide, can be measured (not shown). The rate of reduction of dithionitrobenzoic acid can be used to account for disulfide reduction capacity of compounds under study.

### 2.2.5 Substrates and substrate specificity of HIF prolyl hydroxylases

The data from the literature and that obtained in this laboratory unequivocally demonstrate that PHDs are important targets for medical intervention. This justifies the necessity of the development of HTS for PHD inhibitors, activators, specific and alternative substrates. The challenge is to develop inhibitors specific for each isoform, since very recently it became clear that the PHD isozymes have their specific substrates.

HIF1 and HIF2 are established substrates for PHD2. PHD1 apparently is specific for Rpb1, the large subunit of RNA polymerase II, which carries the fundamental enzymatic activity



of the complex synthesizing all cellular mRNAs. Rpb1 is ubiquitinated and degraded in response to DNA lesions induced by UV light and high millimolar  $H_2O_2$ . Phosphorylation of Ser5 in Rpb1 is a prerequisite for Rpb1 ubiquitination. It has been discovered that Pro1465 hydroxylation catalyzed by PHD1 is necessary for subsequent Ser5 phosphorylation of Rpb1 in response to oxidative stress. PHD2, in contrast, has an inhibitory effect on this modification (Mikhailova et al 2008). Recently, the Kaelin's group (Zhang et al 2009) demonstrated a link between PHD1 and cyclin D1: PHD1 is estrogen-inducible in breast carcinoma cells and PHD1 inactivation decreases Cyclin D1 levels and suppresses mammary gland proliferation *in vivo*. Regulation of Cyclin D1 is a specific attribute of PHD1 among the PHD proteins and is HIF-independent. Loss of PHD1 (but not PHD2) catalytic activity inhibits estrogen-dependent breast cancer tumorigenesis and can be rescued by exogenous Cyclin D1. PHD1 depletion also impairs the fitness of lung, brain, and hematopoietic cancer lines. These findings support the exploration of PHD1 inhibitors as therapeutics for estrogen-dependent breast cancer and other malignancies. PHD1 appears to be an attractive drug target because PHD1 is not essential in mammals.

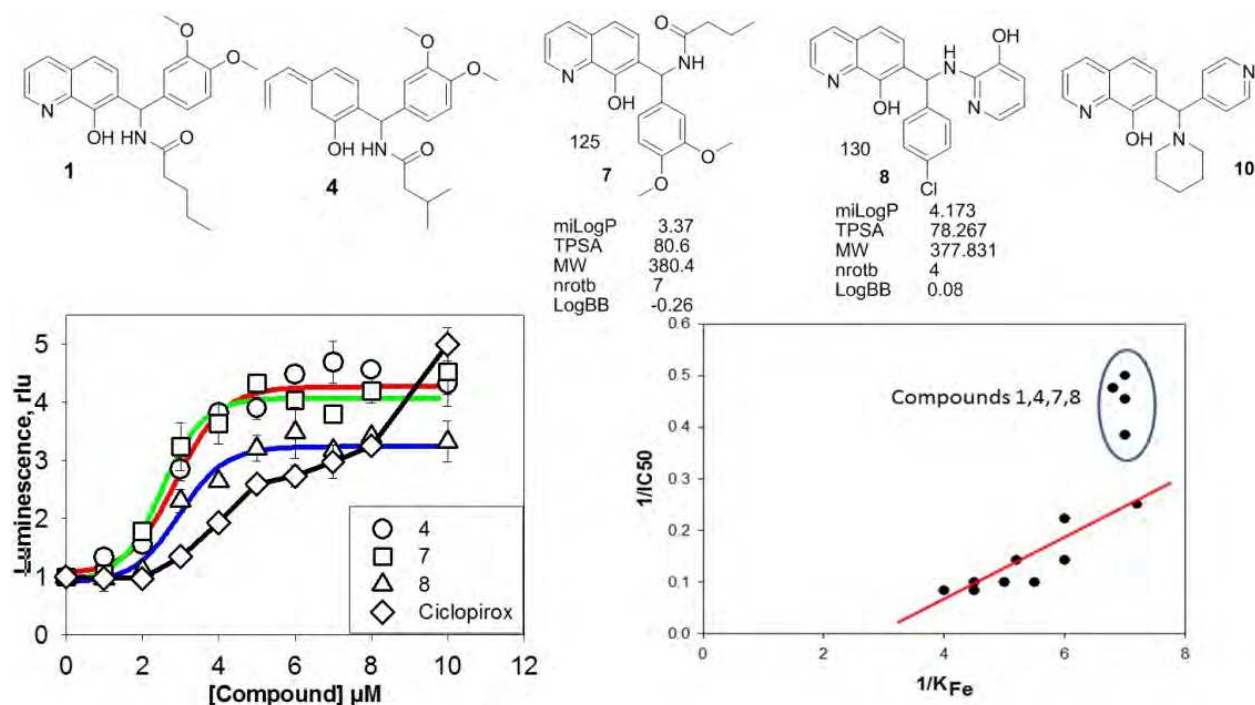


Fig. 14. Evaluation of iron chelation effect on inhibition parameters determined for branched oxyquinolines. Among all compounds of this group tested, only four (compounds 1,4,7,8) showed significant deviation from a linear dependence, and thus are considered as specific PHD inhibitors.

PHD3 hydroxylates  $\beta 2$ -adrenergic receptor ( $\beta 2AR$ ) (Xie et al 2009), the prototypic GPCR that play an important role in the regulation of cardiovascular and pulmonary function, and sustained  $\beta AR$  down-regulation (and dysfunction) is associated with diseases such as heart failure and asthma.  $\beta 2AR$ , in particular, enhances bronchodilation and alveolar fluid clearance (which increase  $O_2$  uptake), enhances cardiac output and peripheral vasodilation (which increase  $O_2$  delivery), and enhances cardioprotection and angiogenesis under

ischemic conditions, thereby effectively recapitulating the integrated physiological response to hypoxia. Up-regulation of the  $\beta$ 2AR in response to hypoxia puts the function of the receptor in new light. The ability of the PHD3-pVHL hydroxylation and ubiquitylation pathway to regulate the  $\beta$ 2AR and the implications of that regulation for the response to ischemia and hypoxia suggest previously unidentified targets in the treatment of cardiovascular and respiratory diseases. PHD3 is most abundant in cardiac and smooth muscle, where the  $\beta$ 2AR is highly abundant *in vivo*. PHD3 expression is increased with aging.

On one hand, the disadvantage of the cell-based assay is that there are 3 different isoforms of the enzyme, and reporter activation shows an integral inhibition of all three or the two most abundant (PHD1 and PHD2). On the other hand, the enzymatic assay is sensitive to the isoform-specificity only when the full-size protein substrate (HIF1) is used. The combination of cell-based HIF1 ODD-luc reporter assay with siRNA technique can give an answer on isoform specificity with respect to HIF1 and can be used to clarify the specificity of a particular drug candidate with respect to PHD isoform.

Cell-based HTS is close to the actual reaction conditions in the cell, where concentration of HIF is at below nanomolar level and thus, permits selection of mild inhibitors. Computerized analysis of hits in the case of PHD inhibitors groups hits in accord with their core structure, which obviously is just a mimic of  $\alpha$ KG. The average number of hits from 10,000 compound library is on the order of 30-40 compounds, and this low number (in comparison with Neh2-luc screen) permits manual classification. The comparison of the structures of various hit groups from HTS screen of 85,000 compounds demonstrated (1) the presence of a branched motif immediately attached to the iron chelation/coordination core, and (2) the effect of the linker length. As shown in Fig.15, long or rigid branch, immediately attached to the iron-coordinating core, has a profound effect on HIF1 ODD-luc reporter activation. In the case of branched oxyquinolines, we were able to study structure-activity relationship in detail (Smirnova et al 2010), and to demonstrate the specific character of PHD inhibition by branched oxyquinolines (see compound 7 in Fig.12 & compounds 1,4,7,8 in Fig. 14).

Structural analysis of the hits comprised of branched quinolines revealed that the general scaffold consists of three regions: the iron binding motif, linker, and the terminal groups that may serve for surface recognition among the various PHD isozymes (Figure 16). Chemical modification on each of these regions will create a substantial molecular space for exploring the pharmacophoric requirements for PHD inhibitory activity and isoform selectivity with the aim to identify the advanced analogues with requisite physiochemical properties.

Our current research is focused on optimization of the branched portion of 8-hydroxyquinolines to develop isoform-specific PHD inhibitors, as shown in Fig.16. Iron-binding groups can be represented by various iron-coordinating cores, and oxyquinolines are just an example chosen based on the ease of synthetic manipulations. As the iron-binding groups can also contribute to the isozyme selectivity, one may investigate structural alterations in the portion that bears an Fe(II)-binding group by analogy with the known structures of PHD inhibitors mimicking ketoglutarate binding (see Fig.17D). It is known that less flexible structures can mimic bound conformation at the target binding site; moreover, structural rigidification reduces CYP450 interactions and hERG blocking activity, therefore allowing for improvement of ADMET characteristics of the proposed inhibitors. As could be envisioned, the core structure of 8-hydroxyquinoline might be constrained by incorporating

the 3,4-dihydrobenzoxazine ring, formed by connecting the 8-hydroxygroup and the nitrogen in the side chain. Notably, a few compounds of that type were defined by initial HTS as the substituted 3,4-dihydro-2*H*-pyrido[3,2-*h*]-1,3-benzoxazine shown in Fig. 17.

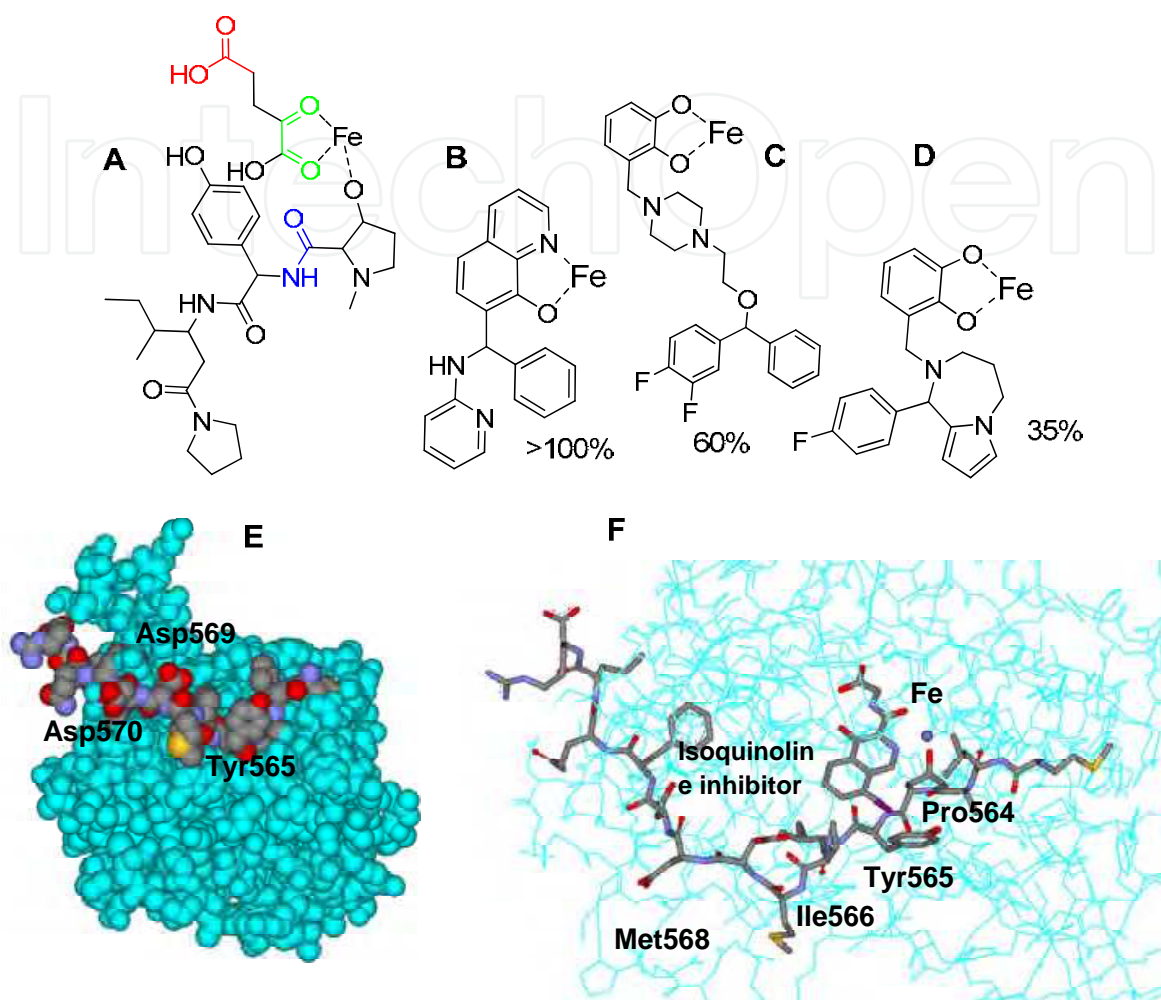


Fig. 15. Identification of branched hits mimicking PHD interaction with HIF peptide. Schematic representation of interaction mode of PYIP portion of HIF peptide with PHD2 active site iron (A) and HTS hits of oxyquinoline group (B) and catechol group (C and D). Docking of HIF peptide into PHD2: (E) Overall view of hydroxylated HIF peptide docked into the PHD2 structure; (F) HIF peptide position with respect to the bound isoquinoline inhibitor. Docking studies have been performed using the available crystal structure of PHD2 (2G19) and hydroxylated HIF peptide as is, in its conformation in the complex with VHL (1LM8.pdb). The only restrain was the orientation and distance of Pro 564 hydroxyl oxygen from iron. The LAPYIP sequence fits into the active site entrance, while the rest of the C-terminal tail goes under the so called  $\beta 2\beta 3$  loop. No minimization was performed on protein. The move of the loop down to cover HIF peptide is the predicted protein minimization result. Tyr565 and Ile566 are located just under the isoquinoline ring plane.

Carboxy-group of FG-0041 interacts with active site Arg-383 in addition to chelation of iron. Benzoxazine hits chelate iron in the active site of PHD the same way. Reporter activation is sensitive to the length of a linker: the latter should be rather flexible and not long. Docking studies show that the branched portion mimics the position of PYIP sequence of HIF

peptide, and thus playing with this portion one can develop inhibitors specifically interacting with the entrance to the PHD active site. Such inhibitors will be able to discriminate between different PHD isoforms.

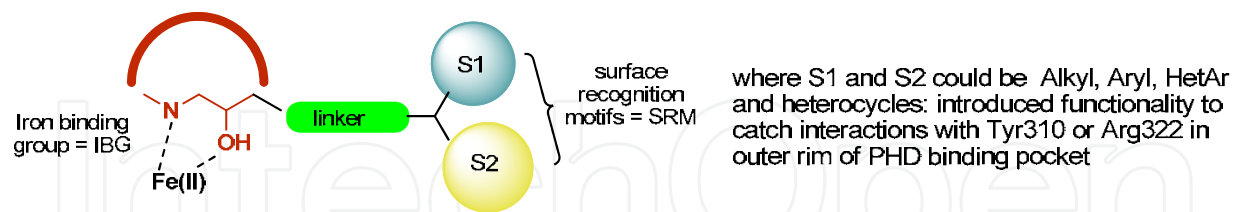


Fig. 16. Variations in chemical structure of PHD inhibitors to improve interaction with the entrance to the enzyme active site.

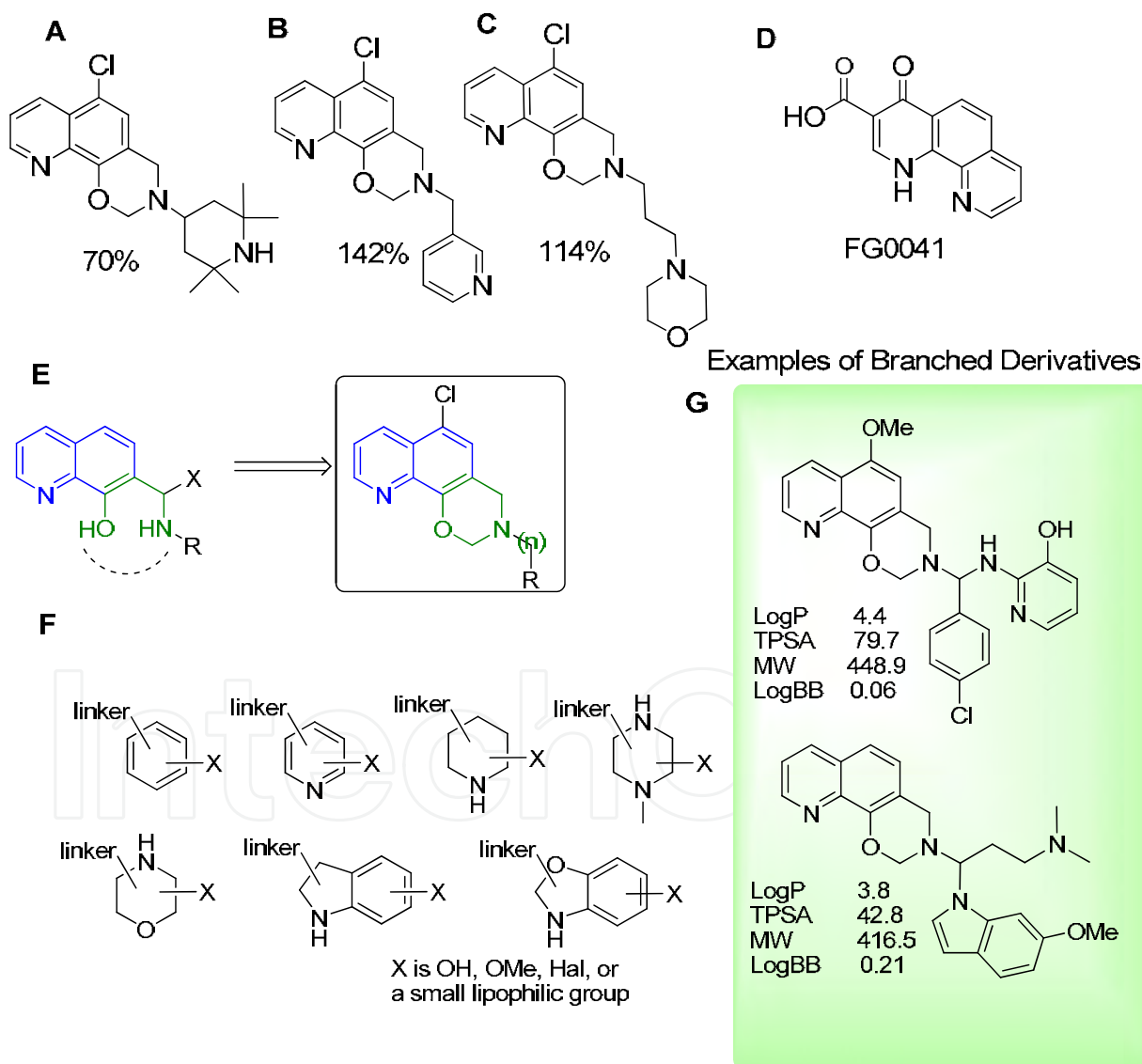


Fig. 17. Exploring 3-Substituted 3,4-dihydro-2H-pyrido[3,2-h]-1,3-benzoxazine hits from HTS (A-C). D: Fibrogen developed PHD inhibitor (FG-0041). E-F: Variations in the branched portion of benzoxazines.



### 3. Conclusion

The novel approach to HTS of transcription factor activators is extremely promising for the development of specific stabilizers of the corresponding transcription factor, because it is sensitive to the initial step in transcription factor stabilization. The magnitude and sensitivity of reporter assay permits selection of mild activators which provide “fine tuning”. The key characteristic of new reporter systems is the possibility to monitor reporter activation in real-time to support structure-activity relationship studies (see Figs. 4 & 11). No other reporter provides such a possibility, and no other reporter provides such sensitivity. For both reporters discussed, this new feature opens a way towards design of specific stabilizers of the corresponding transcription factor.

We were first to predict the possibility of development of isoform-specific PHD inhibitors employing variations in the motif adjacent to the iron binding core based on the results of HTS (Smirnova et al 2010). This prediction was supported by the identified branched oxyquinolines, which were characterized as PHD specific inhibitors and were confirmed to exert the predicted biological effects. Our conclusion on the role of branched portion in recognition of different PHD isoforms has been experimentally confirmed in the recent publication from Amgen group: the branching peptide-like portions attached to the quinolone core (see Compound 12{1,1,2} in Fig.9) resulted in up to 10-fold difference in magnitude of inhibition of PHD2 and PHD1/PHD3 (Murray et al 2010). Hence, isoform-specific PHD inhibitors can be constructed by optimizing the branching portion immediately adjacent to the iron-chelation core. Ongoing work includes synthesis of branched derivatives (Fig. 17) to explore the possibility of developing isoform-specific inhibitors.

We were first to demonstrate the direct effect of gedunins on Nrf2 activation as well as their perfect docking into Keap1 in place of Neh2 peptide. The latter discovery is promising in terms of future identification of non-electrophilic activators working by displacing of Nrf2 from its complex with Keap1, without non-specific oxidation or covalent modification of Keap thiols among other cellular proteins.

The collection of reporters available in the laboratory (HIF1 ODD-luc and its mutants, HIF2 ODD-luc, HIF3 ODD-luc, Neh2-luc) permits their parallel use for HTS purposes, where each reporter plays the role of a control for each other. The new reporter assays combined with secondary biochemical analysis permit selection of drugs working at the first, selective step of transcription factor stabilization.

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### 5. References

- Allen, J. R.; Tegley, C. M.; Biswas, K.; Burli, R.; Muller, K. M.; Frohn, M. J.; Golden, J. E.; Mercede, S. J.; Neira, S. C.; Peterkin, T. A. N.; Hungate, R. W.; Kurzeja, R.; Yu, V.; Dao, J. Quinolone based compounds exhibiting prolyl hydroxylase inhibitory activity, and compositions, and uses thereof. WO Patent 2007070359, Apr 3, 2008.

- Baell, J.B., and Holloway, G.A. (2010). New substructure filters for removal of pan assay interference compounds (PAINS) from screening libraries and for their exclusion in bioassays. *J Med Chem* 53, 2719-2740.
- Balani SK, Miwa GT, Gan LS, Wu JT, Lee FW. (2005). Strategy of utilizing in vitro and in vivo ADME tools for lead optimization and drug candidate selection. *Curr. Topics Med. Chem.* 5, 1033-1038
- Bruick, R.K., and McKnight, S.L. (2001). A conserved family of prolyl-4-hydroxylases that modify HIF. *Science* 294, 1337-1340.
- Cho, H., Park, H., and Yang, E.G. (2005). A fluorescence polarization-based interaction assay for hypoxia-inducible factor prolyl hydroxylases. *Biochem. Biophys. Res. Commun.* 337, 275-280.
- Chowdhury, R., McDonough, M.A., Mecinovic, J., Loenarz, C., Flashman, E., Hewitson, K.S., Domene, C., and Schofield, C.J. (2009). Structural basis for binding of hypoxia-inducible factor to the oxygen-sensing prolyl hydroxylases. *Structure* 17, 981-989.
- Cullinan, S.B., Gordan, J.D., Jin, J., Harper, J.W., and Diehl, J.A. (2004). The Keap1-BTB protein is an adaptor that bridges Nrf2 to a Cul3-based E3 ligase: oxidative stress sensing by a Cul3-Keap1 ligase. *Mol. Cell. Biol.* 24, 8477-8486.
- Dao, J.H., Kurzeja, R.J., Morachis, J.M., Veith, H., Lewis, J., Yu, V., Tegley, C.M., and Tagari, P. (2009). Kinetic characterization and identification of a novel inhibitor of hypoxia-inducible factor prolyl hydroxylase 2 using a time-resolved fluorescence resonance energy transfer-based assay technology. *Anal. Biochem.* 384, 213-223.
- Hewitson, K.S., Schofield, C.J., and Ratcliffe, P.J. (2007). Hypoxia-inducible factor prolyl-hydroxylase: purification and assays of PHD2. *Methods Enzymol.* 435, 25-42.
- Hirotsu, Y., Katsuoka, F., Itoh, K., and Yamamoto, M. (2011). Nrf2 degron-fused reporter system: a new tool for specific evaluation of Nrf2 inducers. *Genes Cells* 16, 406-415.
- Ivan, M., Haberberger, T., Gervasi, D.C., et al. (2002). Biochemical purification and pharmacological inhibition of a mammalian prolyl hydroxylase acting on hypoxia-inducible factor. *Proc. Natl. Acad. Sci. USA* 99, 13459-13464.
- Itoh, K., Wakabayashi, N., Katoh, Y., Ishii, T., Igarashi, K., Engel, J.D., and Yamamoto, M. (1999). Keap1 represses nuclear activation of antioxidant responsive elements by Nrf2 through binding to the amino-terminal Neh2 domain. *Genes Dev.* 13, 76-86.
- Kaelin, W.G., Jr. (2005). The von Hippel-Lindau protein, HIF hydroxylation, and oxygen sensing. *Biochem. Biophys. Res. Commun.* 338, 627-638.
- Kaspar, J.W., Niture, S.K., and Jaiswal, A.K. (2009). Nrf2:INrf2 (Keap1) signaling in oxidative stress. *Free Radic. Biol. Med.* 47, 1304-1309.
- Kobayashi, A., Kang, M.I., Okawa, H., Ohtsuji, M., Zenke, Y., Chiba, T., Igarashi, K., and Yamamoto, M. (2004). Oxidative stress sensor Keap1 functions as an adaptor for Cul3-based E3 ligase to regulate proteasomal degradation of Nrf2. *Mol. Cell. Biol.* 24, 7130-7139.
- Koivunen, P., Hirsila, M., Kivirikko, K.I., and Myllyharju, J. (2006). The length of peptide substrates has a marked effect on hydroxylation by the hypoxia-inducible factor prolyl 4-hydroxylases. *J. Biol. Chem.* 281, 28712-28720.
- Kola I, Landis J. (2004). Can the pharmaceutical industry reduce attrition rates? *Nat. Rev. Drug Discov.* 3, 711-715
- Komatsu, M., Kurokawa, H., Waguri, S., Taguchi, K., Kobayashi, A., Ichimura, Y., Sou, Y.S., Ueno, I., Sakamoto, A., Tong, K.I., et al. (2010). The selective autophagy substrate

- p62 activates the stress responsive transcription factor Nrf2 through inactivation of Keap1. *Nat. Cell Biol.* 12, 213–223.
- Landazuri, M.O., Vara-Vega, A., Viton, M., Cuevas, Y., and del Peso, L. (2006). Analysis of HIF-prolyl hydroxylases binding to substrates. *Biochem. Biophys. Res. Commun.* 351, 313–320.
- Mikhaylova, O., Ignacak, M.L., Barankiewicz, T.J., Harbaugh, S.V., Yi, Y., Maxwell, P.H., Schneider, M., Van Geyte, K., Carmeliet, P., Revelo, M.P., et al. (2008). The von Hippel-Lindau tumor suppressor protein and Egl-9-Type proline hydroxylases regulate the large subunit of RNA polymerase II in response to oxidative stress. *Mol. Cell. Biol.* 28, 2701–2717.
- Moi, P., Chan, K., Asunis, I., Cao, A., and Kan, Y.W. (1994). Isolation of NF-E2-related factor 2 (Nrf2), a NF-E2-like basic leucine zipper transcriptional activator that binds to the tandem NF-E2/AP1 repeat of the beta-globin locus control region. *Proc. Natl. Acad. Sci. USA* 91, 9926–9930.
- Moehlenkamp, J.D., and Johnson, J.A. (1999). Activation of antioxidant/electrophile-responsive elements in IMR-32 human neuroblastoma cells. *Arch. Biochem. Biophys.* 363, 98–106.
- Motohashi, H., and Yamamoto, M. (2004). Nrf2-Keap1 defines a physiologically important stress response mechanism. *Trends Mol. Med.* 10, 549–557.
- Murray, J.K., Balan, C., Allgeier, A.M., Kasparian, A., Viswanadhan, V., Wilde, C., Allen, J.R., Yoder, S.C., Biddlecome, G., Hungate, R.W., Miranda, L.P. (2010) Dipeptidyl-quinolone derivatives inhibit hypoxia inducible factor-1 $\alpha$  prolyl hydroxylases-1, -2, and -3 with altered selectivity. *J Comb Chem.* 12(5), 676–686.
- Nangaku, M., Izuhara, Y., Takizawa, S., Yamashita, T., Fujii-Kuriyama, Y., Ohneda, O., Yamamoto, M., van Ypersele de Strihou, C., Hirayama, N., and Miyata, T. (2007). A novel class of prolyl hydroxylase inhibitors induces angiogenesis and exerts organ protection against ischemia. *Arterioscler. Thromb. Vasc. Biol.* 27, 2548–2554.
- Price, J.C., Barr, E.W., Hoffart, L.M., Krebs, C., and Bollinger, J.M., Jr. (2005). Kinetic dissection of the catalytic mechanism of taurine:alpha-ketoglutarate dioxygenase (TauD) from *Escherichia coli*. *Biochemistry* 44, 8138–8147.
- Safran, M., Kim, W.Y., O'Connell, F., Flippin, L., Gunzler, V., Horner, J.W., Depinho, R.A., and Kaelin, W.G., Jr. (2006). Mouse model for noninvasive imaging of HIF prolyl hydroxylase activity: assessment of an oral agent that stimulates erythropoietin production. *Proc. Natl. Acad. Sci. USA* 103, 105–110.
- Semenza, G.L., Jiang, B.H., Leung, S.W., Passantino, R., Concordet, J.P., Maire, P., and Giallongo, A. (1996). Hypoxia response elements in the aldolase A, enolase 1, and lactate dehydrogenase A gene promoters contain essential binding sites for hypoxia-inducible factor 1. *J. Biol. Chem.* 271, 32529–32537.
- Shaw, P., Mead, R., Higginbottom, A., and Barber, S. (2010) Therapeutics for neurological disorders. UK Patent Application #0918626.3 Priority Date 24.10.2008. Publ Date 05.05.2010.
- Smirnova, N.A., Rakhman, I., Moroz, N., Basso, M., Payappilly, J., Kazakov, S., Hernandez-Guzman, F., Gaisina, I.N., Kozikowski, A.P., Ratan, R.R., et al. (2010). Utilization of an in vivo reporter for high throughput identification of branched small molecule regulators of hypoxic adaptation. *Chem. Biol.* 17, 380–391.

- Smirnova, N.A., Haskew-Layton, R.E., Basso, M., Hushpulian, D.M., Payappilly, J.B., Speer, R.E., Ahn, Y.H., Rakhman, I., Cole, P.A., Pinto, J.T., Ratan, R.R., and Gazaryan, I.G. (2011). Development of *neh2*-luciferase reporter and its application for high throughput screening and real-time monitoring of *nrf2* activators. *Chem Biol* 18, 752-765.
- Solomon, E.I., Brunold, T.C., Davis, M.I., Kemsley, J.N., Lee, S.K., Lehnert, N., Neese, F., Skulan, A.J., Yang, Y.S., and Zhou, J. (2000). Geometric and electronic electronic structure/function correlations in non-heme iron enzymes. *Chem. Rev.* 100, 235-350.
- Tegley, C.M., Viswanadhan, V.N., Biswas, K., Frohn, M.J., Peterkin, T.A., Chang, C., Bu' rli, R.W., Dao, J.H., Veith, H., Rogers, N., et al. (2008). Discovery of novel hydroxythiazoles as HIF- $\alpha$  prolyl hydroxylase inhibitors: SAR, synthesis, and modeling evaluation. *Bioorg. Med. Chem. Lett.* 18, 3925-3928.
- Thompson TN. (2000). Early ADME in support of drug discovery: the role of metabolic stability studies. *Curr. Drug Metab.* 1, 215-241.
- Tong, K.I., Katoh, Y., Kusunoki, H., Itoh, K., Tanaka, T., and Yamamoto, M. (2006a). Keap1 recruits Neh2 through binding to ETGE and DLG motifs: characterization of the two-site molecular recognition model. *Mol. Cell. Biol.* 26, 2887-2900.
- Tong, K.I., Kobayashi, A., Katsuoka, F., and Yamamoto, M. (2006b). Two-site substrate recognition model for the Keap1-Nrf2 system: a hinge and latch mechanism. *Biol. Chem.* 387, 1311-1320.
- Tuckerman, J.R., Zhao, Y., Hewitson, K.S., Tian, Y.M., Pugh, C.W., Ratcliffe, P.J., and Mole, D.R. (2004). Determination and comparison of specific activity of the HIF-prolyl hydroxylases. *FEBS Lett.* 576, 145-150.
- Wang, G.L., Jiang, B.H., Rue, E.A., and Semenza, G.L. (1995). Hypoxia-inducible factor 1 is a basic-helix-loop-helix-PAS heterodimer regulated by cellular O<sub>2</sub> tension. *Proc. Natl. Acad. Sci. USA* 92, 5510-5514.
- Warshakoon, N.C., Wu, S., Boyer, A., Kawamoto, R., Renock, S., Xu, K., Pokross, M., Evdokimov, A.G., Zhou, S., et al. (2006a). Design and synthesis of a series of novel pyrazolopyridines as HIF-1 $\alpha$  prolyl hydroxylase inhibitors. *Bioorg. Med. Chem. Lett.* 16, 5687-5690.
- Warshakoon, N.C., Wu, S., Boyer, A., Kawamoto, R., Sheville, J., Bhatt, R.T., Renock, S., Xu, K., Pokross, M., Zhou, S., et al. (2006b). Design and synthesis of substituted pyridine derivatives as HIF-1 $\alpha$  prolyl hydroxylase inhibitors. *Bioorg. Med. Chem. Lett.* 16, 5616-5620.
- Warshakoon, N.C., Wu, S., Boyer, A., Kawamoto, R., Sheville, J., Renock, S., Xu, K., Pokross, M., Evdokimov, A.G., Walter, R., and Mekel, M. (2006c). A novel series of imidazo[1,2-a]pyridine derivatives as HIF-1 $\alpha$  prolyl hydroxylase inhibitors. *Bioorg. Med. Chem. Lett.* 16, 5598-5601.
- Warshakoon, N.C., Wu, S., Boyer, A., Kawamoto, R., Sheville, J., Renock, S., Xu, K., Pokross, M., Zhou, S., Winter, C., et al. (2006d). Structure-based design, synthesis, and SAR evaluation of a new series of 8-hydroxyquinolines as HIF-1 $\alpha$  prolyl hydroxylase inhibitors. *Bioorg. Med. Chem. Lett.* 16, 5517-5522.
- Wu, J.H., Miao, W., Hu, L.G., and Batist, G. (2010) Identification and characterization of novel Nrf2 inducers designed to target the intervening region of Keap1. *Chem. Biol. Drug Des.* 75, 475-480.



- Xie, L., Xiao, K., Whalen, E.J., Forrester, M.T., Freeman, R.S., Fong, G., Gygi, S.P., Lefkowitz, R.J., and Stamler, J.S. (2009). Oxygen-regulated 2-adrenergic receptor hydroxylation by EGLN3 and ubiquitylation by pVHL. *Sci. Signal* 2, 1–10.
- Zhang, D.D. (2006). Mechanistic studies of the Nrf2-Keap1 signaling pathway. *Drug Metab. Rev.* 38, 769–789.
- Zhang, D.D., Lo, S.C., Cross, J.V., Templeton, D.J., and Hannink, M. (2004). Keap1 is a redox-regulated substrate adaptor protein for a Cul3-dependent ubiquitin ligase complex. *Mol. Cell. Biol.* 24, 10941–10953.
- Zhang, Q., Gu, J., Li, L., Liu, J., Luo, B., Cheung, H.W., Boehm, J.S., Ni, M., Geisen, C., Root, D.E., Polyak, K., Brown, M., Richardson, A.L., Hahn, W.C., Kaelin, W.G. Jr, Bommi-Reddy, A. (2009) Control of cyclin D1 and breast tumorigenesis by the EglN2 prolyl hydroxylase. *Cancer Cell* 16(5), 413–24.

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## **Drug Discovery and Development - Present and Future**

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Drug discovery and development process aims to make available medications that are safe and effective in improving the length and quality of life and relieving pain and suffering. However, the process is very complex, time consuming, resource intensive, requiring multi-disciplinary expertise and innovative approaches. There is a growing urgency to identify and develop more effective, efficient, and expedient ways to bring safe and effective products to the market. The drug discovery and development process relies on the utilization of relevant and robust tools, methods, models, and validated biomarkers that are predictive of clinical effects in terms of diagnosis, prevention, therapy, and prognosis. There is a growing emphasis on translational research, a bidirectional bench to the bedside approach, in an effort to improve the process efficiency and the need for further innovations. The authors in the book discuss the current and evolving state of drug discovery and development.

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